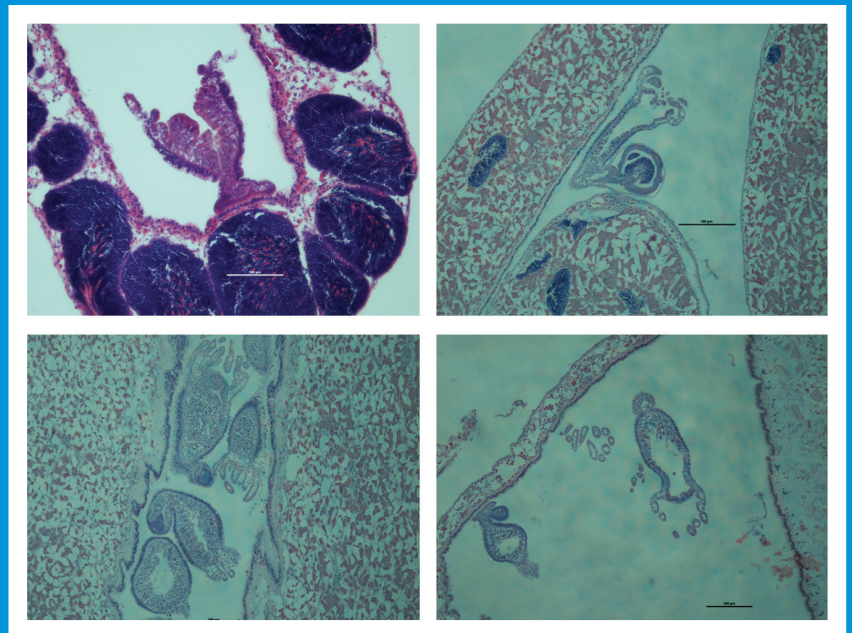


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# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK



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# PREVALENCE OF *CAMPYLOBACTER* SPECIES IN FECAL SAMPLES FROM CATS AND DOGS IN IRAN

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**Summary:** *Campylobacter* spp. are one of the most frequent pathogens of acute bacterial gastroenteritis in human beings. The present study was conducted to determine the prevalence of *Campylobacter* spp. from dog and cat fecal samples in Iran. From August 2010 to August 2011, a total of 173 samples of fresh feces from pet dogs (n = 126) and cats (n = 47) were collected by the owners in Fars and Isfahan provinces, Iran. In this study, 61 of 173 fecal samples (35.3%) were found to be contaminated with *Campylobacter*. *Campylobacter* spp. were isolated from 48 dogs (38.1%) and from 13 cats (27.7%). Twenty-five *C. upsaliensis*, 18 *C. jejuni*, 5 *C. coli* isolates from dogs and 1 *C. upsaliensis*, 8 *C. helveticus*, 4 *C. jejuni*, isolates from cats were identified using both the cultural method and the PCR assay. The prevalence of *Campylobacter* in adult dogs (29.6%) was lower than in young dogs (49.1%). However, there were not significant differences in the prevalence of *Campylobacter* between adult (29.4%) and juveniles cats (26.7%). Also, no statistically significant correlation was found between the isolation of *Campylobacter*, and the presence of gastroenteric disorders, in either dogs or cats. To the authors' knowledge, the present study is the first report on the prevalence of *Campylobacter* in dogs and cats in Iran.

**Key words:** dogs; *Campylobacter*; cats; zoonosis

## Introduction

The family Campylobacteriaceae comprises small, spiral form, Gram-negative bacteria with 25 species and 11 sub-species (1, 2). They are essentially microaerophilic, growing best in an atmosphere containing approximately 10% CO<sub>2</sub> and approximately 5% O<sub>2</sub>. *Campylobacter* species, in particular *C. jejuni* and *C. coli*, are considered to be the most frequent bacterial cause of human enteritis but in a small proportion of cases *C. upsaliensis* has been reported (3, 4). *Campylobacter* species are widely distributed in nature and have been associated with poultry, pigs, cattle, sheep, shellfish, dogs and cats (5, 6). Consumption of undercooked meat, unpasteurized milk, and contaminated drinking water is considered an important risk factor for campylobacteriosis (7, 8).

Cross-contamination of ready to eat foods during food preparations with *Campylobacter* spp. as well as direct contact with pet animals have been reported (4, 8). There is evidence of increased risk of *Campylobacter* infection in humans associated with dog or pet ownership (6, 9) with studies indicating an association between *C. jejuni* (10), and *C. upsaliensis* (11) infection in humans and dogs in the same household. Dogs are regarded as important reservoir for *C. upsaliensis*, and cats were shown to be carriers of *C. helveticus* (5, 12) a thermophilic *Campylobacter* species which is difficult to differentiate from *C. upsaliensis* by biochemical tests (5, 12). The development of more sensitive detection methods has allowed for more accurate detection, isolation, and classification of *Campylobacter* spp. These advances in surveillance technology have provided improved information on the prevalence of *Campylobacter* spp. worldwide and now demonstrate that this

pathogen can be interspecies specific rather than just limited to warm blooded hosts as was once thought (5).

Such information is important for epidemiological purposes and could help in assessing the role of *Campylobacter* as a pathogen in these animals. *Campylobacter* has been reported in dogs and cats in some countries of the world (2, 4-12) and campylobacters in cat and dog populations are of concern for the animals themselves and for members of the public on account of the possible risks of zoonotic infection. Currently, there is limited information regarding the prevalence of *Campylobacter* in pet animals in Iran. The present study was conducted to determine the prevalence of *Campylobacter* spp. in dog and cat fecal samples in Fars and Isfahan provinces, Iran.

## Materials and methods

### *Sample collection*

From August 2010 to August 2011, a total of 173 samples of fresh feces from pet dogs (n = 126) and cats (n = 47) were collected by the owners in Fars and Isfahan provinces, Iran. All samples were placed in separate sterile plastic bags to prevent spilling and cross contamination and were immediately transported to the laboratory in a cooler with ice packs. Age distribution of animals was as follows: 71 dogs were adult (>12 months), 55 dogs were younger than 1 year. Cat samples were obtained from 17 adult and 30 juveniles were provided. Diarrhea was reported in 38 dogs and 11 cats. The remaining animals had no clinical signs reported by their owner.

### *Microbiological analysis*

The samples were processed immediately upon arrival and at latest six hours after sampling, using aseptic techniques. Approximately 5 g of feces were homogenized in 45 ml of Preston enrichment broth base containing *Campylobacter* selective supplement IV (HiMedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood. After inoculation at 42 °C for 24 h in a microaerophilic condition (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% O<sub>2</sub>), 0.1 mL of the enrichment was then streaked onto Preston selective agar base (HiMedia Laboratories, Mumbai, India) supplemented with an antibiotic supplement for the selective isolation

of *Campylobacter* species (HiMedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood and incubated at 42 °C for 48 h under the same condition. One presumptive *Campylobacter* colony from each selective agar plate was subcultured and identification of presumptive *Campylobacter* species was performed using standard microbiological and biochemical procedures including Gram staining, production of catalase, oxidase, hippurate hydrolysis, urease activity, indoxyl acetate hydrolysis, growth in the presence of 1% (w/v) glycine and 0.04% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC), H<sub>2</sub>S production in triple sugar iron (TSI) agar and susceptibility to cephalotin (13, 14).

### *DNA extraction and identification of Campylobacter species*

Only *Campylobacter* spp. isolates identified by bacteriological methods were tested by PCR. Briefly, 1 mL of pure culture of *Campylobacter* was centrifuged at 13000 g for 5 min at room temperature. The DNA was then extracted using a genomic DNA purification kit (Fermentas, GmbH, Germany, K0512) according to the manufacturer's protocol. The isolates underwent genus specific PCRs for *Campylobacter* (15). The isolates were identified at the species level by *C. jejuni*, and *C. coli* specific multiplex PCR (16), *C. upsaliensis*, and *C. helveticus* specific duplex PCR (17).

### *Statistical analysis*

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), chi-square test and fisher's exact two-tailed test analysis were performed and differences were considered significant at values of P < 0.05.

## Results

Table 1 shows the prevalence of *Campylobacter* spp. isolated from 173 samples of fresh feces from pet dogs and cats in Fars and Isfahan provinces, Iran. Overall, 61 of 173 fecal samples (35.3%) were positive for *Campylobacter* spp. using both the cultural method and the PCR assay. *Campylobacter* spp. were isolated from 48 dogs

**Table 1:** Prevalence of *Campylobacter* spp. from dogs and cats using both the cultural method and the PCR assay

Samples	No. of samples	<i>Campylobacter</i> spp. positive*	<i>C. upsaliensis</i>	<i>C. helveticus</i>	<i>C. jejuni</i>	<i>C. coli</i>
Dogs	126	48 (38.1) <sup>a</sup>	25 (52.1) <sup>a</sup>	0 (0.0) <sup>a</sup>	18 (37.5) <sup>a</sup>	5 (10.6) <sup>a</sup>
Cats	47	13 (27.7) <sup>b</sup>	1 (7.7) <sup>b</sup>	8 (61.5) <sup>b</sup>	4 (30.8) <sup>a</sup>	0 (0.0) <sup>b</sup>
Total	173	61 (35.3)	26 (42.6)	8 (13.1)	22 (36.1)	5 (8.2)

\* Results expressed as the number of *Campylobacter*-positive samples / number of samples analyzed (%).

<sup>a, b</sup> Values in the same column with different superscripts are significantly different ( $P < 0.05$ ).

**Table 2:** Prevalence of *Campylobacter* spp. isolated from healthy and diarrheic dogs and cats using both the cultural method and the PCR assay

Samples	No. of samples	<i>Campylobacter</i> spp. positive*	<i>C. upsaliensis</i>	<i>C. helveticus</i>	<i>C. jejuni</i>	<i>C. coli</i>	
Dogs	Healthy	88	19 (52.8) <sup>*a</sup>	0 (0.0) <sup>a</sup>	13 (36.1) <sup>a</sup>	13 (36.1) <sup>a</sup>	4 (11.1) <sup>a</sup>
	Diarrhoeic	38	6 (50.0) <sup>a</sup>	0 (0.0) <sup>a</sup>	5 (41.7) <sup>a</sup>	5 (41.7) <sup>a</sup>	1 (8.3) <sup>a</sup>
Cats	Healthy	36	0 (0.0) <sup>a</sup>	7 (70.0) <sup>a</sup>	3 (30.0) <sup>a</sup>	3 (30.0) <sup>a</sup>	0 (0.0) <sup>a</sup>
	Diarrhoeic	11	1 (33.3) <sup>b</sup>	1 (33.3) <sup>b</sup>	1 (33.3) <sup>a</sup>	1 (33.3) <sup>a</sup>	1 (33.3) <sup>b</sup>

\* Results expressed as the number of *Campylobacter*-positive samples / number of samples analyzed (%)

<sup>a, b</sup> In each column values with no common superscripts are significantly different ( $P < 0.05$ )

**Table 3:** Prevalence of *Campylobacter* spp. isolated from young and adult dogs and cats using both the cultural method and the PCR assay

Samples	No. of samples	<i>Campylobacter</i> spp. positive	<i>C. upsaliensis</i>	<i>C. helveticus</i>	<i>C. jejuni</i>	<i>C. coli</i>	
Dogs	Adult*	71	21 (29.6) <sup>**a</sup>	9 (42.9) <sup>a</sup>	0 (0.0) <sup>a</sup>	8 (38.1) <sup>a</sup>	4 (19.0) <sup>a</sup>
	Young	55	27 (49.1) <sup>b</sup>	16 (59.3) <sup>a</sup>	0 (0.0) <sup>a</sup>	10 (37.0) <sup>a</sup>	1 (37.0) <sup>b</sup>
Cats	Adult	17	5 (29.4) <sup>a</sup>	1 (20.0) <sup>a</sup>	2 (40.0) <sup>a</sup>	2 (40.0) <sup>a</sup>	0 (0.0) <sup>a</sup>
	Young	30	8 (26.7) <sup>a</sup>	0 (0.0) <sup>b</sup>	6 (75.0) <sup>b</sup>	2 (25.0) <sup>a</sup>	0 (0.0) <sup>a</sup>

\* Adult (> 12 months), Young (< 12 months)

\*\* Results expressed as the number of *Campylobacter*-positive samples / number of samples analyzed (%)

<sup>a, b</sup> In each column values with no common superscripts are significantly different ( $P < 0.05$ )

**Table 4:** Seasonal prevalence of *Campylobacter* spp. isolated from dogs and cats using both the cultural method and the PCR assay

Season	Fecal samples*		Total
	Dogs	Cats	
Summer	17/40 (42.5)	4/14 (28.6)	21/54 (38.9)
Fall	11/38 (36.8)	3/10 (30.0)	14/48 (29.2)
Winter	9/24 (37.5)	2/8 (25.0)	11/32 (34.4)
Spring	11/24 (45.8)	4/15 (26.7)	15/39 (38.5)

\* Results expressed as the number of *Campylobacter*-positive samples / number of samples analyzed (%)

Table 4 shows the seasonal prevalence of *Campylobacter* spp. in dog and cat fecal samples. The highest prevalence of *Campylobacter* spp. occurred in summer (38.9%) followed by spring (38.5%). The prevalence rates of *Campylobacter* spp. in fall and winter were 29.2% and 34.4%, respectively. No significant differences in the prevalence rates of *Campylobacter* spp. were observed for dog and cat fecal samples taken in different seasons in Isfahan, and Fars provinces, Iran.



(38.1%) and from 13 cats (27.7%). There were not significant differences ( $P > 0.05$ ) in the prevalence of *Campylobacter* between different fecal samples. The most prevalent *Campylobacter* species isolated from canine samples was *C. upsaliensis* (52.1%), followed by *C. jejuni* (37.5%) and *C. coli* (10.4%). The most prevalence *Campylobacter* species isolated from cat samples was *C. helveticus* (61.5%); the remaining isolates were *C. jejuni* (30.8%) and *C. upsaliensis* (7.7%). No statistically significant correlation was found between the isolation of *Campylobacter*, and the presence of gastroenteric disorders, in either dogs or cats (Table 2). Also, no significant differences in the prevalence rates of *Campylobacter* spp. were observed between fecal samples isolated in Fars and Isfahan provinces (data not shown). In this study the prevalence of *Campylobacter* in adult dogs (29.6%) was lower than in young dogs (49.1%) ( $P < 0.05$ ). However, there were not significant differences ( $P > 0.05$ ) in the prevalence of *Campylobacter* between adult (29.4%) and juveniles cats (26.7%) (Table 3).

## Discussion

The prevalence rate of *Campylobacter* spp. in dog and cat fecal samples was 38.1% and 27.7% respectively, which is comparable with those reported from Denmark, Norweg, Switzerland, Italy, Nigeria, The UK (3, 6, 12, 18-20); however, higher prevalence rates have been reported by others (21-24). *C. upsaliensis* was the most frequently isolated species in dogs and *C. helveticus* from cats while the isolation rates of *C. jejuni* were similar in both animals. The prevalence of dogs carrying *Campylobacter* spp. varies widely, depending on the population sampled and probably also on the detection methods used (3, 18-20, 23, 25). Frequently, *C. upsaliensis* has been found to be the most common species isolated from dogs (6, 19, 20, 25), although in other studies, *C. jejuni* predominated (18, 26, 27). In any case, cats predominantly carry *C. helveticus* rather than *C. upsaliensis* (3, 20, 22, 26).

When age was investigated as a risk indicator for *Campylobacter* spp. carriage in dogs, the majority of studies found that younger rather than older dogs were more likely to carry *C. upsaliensis* and *C. jejuni* (3, 4, 6, 19, 21, 23). Similar to other studies, we found that younger dogs were more likely to be carriers of *C. upsaliensis* than older dogs and that this is probably a consequence of

age-related immunity. However, a small number of reports have suggested that age is not a risk indicator for *C. jejuni* infection (3, 12, 20, 27). There was no statistically significant association between *Campylobacter* carrier status and clinical history or signs as has been reported by others (6, 19-21, 23, 26); however, higher prevalence rates in diarrheic cat rather than and healthy cat have been reported by Queen et al. (22).

Although various outbreak and seasonal peak of *Campylobacter* have been reported in the warmer months (5), in our study no apparent pattern in the seasonality of *Campylobacter* prevalence was observed. This observation is in agreement with the findings reported by Hudson et al. (7).

The high prevalence of *Campylobacter* carriers found in dogs and cats in this and previous studies suggests the bacteria may be intestinal commensals in this species. Although the relationship between the presence of *C. upsaliensis* and gastroenteritis in both dogs and humans is still unclear, it is worth highlighting that younger dogs in particular may pose a zoonotic risk (4). However the prevalence of *C. jejuni*, the most common *Campylobacter* spp. associated with disease in humans, was the second most common *Campylobacter* species isolated from dogs and cats in our study. To establish the zoonotic potential of canine *Campylobacter* isolates, both human and canine isolates have to be further characterized and compared. To the authors' knowledge, the present study is the first report on the prevalence of *Campylobacter* in dogs and cats in Iran.

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## PREVALENCA BAKTERIJ VRSTE KAMPILOBAKTER V VZORCIH BLATA PSOV IN MAČK V IRANU

E. Rahimi, A. Chakeri, K. Esmizadeh

**Povzetek:** Okužba z bakterijami kampilobakter (*Campylobacter* spp.) je eden izmed najpogostejših povzročiteljev akutnega bakterijskega gastroenteritisa pri ljudeh. Namen naše raziskave je bil določiti razširjenost bakterij *Campylobacter* spp. v iztrebkih psov in mačk v Iranu. Od avgusta 2010 do avgusta 2011 smo s pomočjo lastnikov zbrali 173 vzorcev svežih iztrebkov hišnih psov ( $n = 126$ ) in mačk ( $n = 47$ ) v provincah Fars in Isfahan v Iranu. V 61 vzorcih (35,3 %) smo potrdili prisotnost bakterij kampilobakter, in sicer pri 48 vzorcih psov (38,1 %) in 13 vzorcih mačk (27,7 %). Posamezne vrste bakterij kampilobakter smo določili z mikrobiološko metodo in metodo PCR in pri psih ugotovili *C. upsaliensis* v 25 vzorcih, *C. jejuni* v 18 in *C. coli* v 5. Pri mačkah smo potrdili *C. upsaliensis* v enem vzorcu, *C. helveticus* v 8 in *C. jejuni* v 4 vzorcih. Razširjenost bakterij kampilobakter pri odraslih psih (29,6 %) je bila nižja kot pri mladih (49,1 %), pri mačkah pa ni bilo značilne razlike med odraslimi (29,4 %) in mladimi živalmi (26,7 %). Prav tako ni bilo statistično pomembne povezave med prisotnostjo bakterij kampilobakter v iztrebkih in gastrointestinalnimi motnjami tako pri psih kot pri mačkah. Ta raziskava je prvo poročilo o razširjenosti bakterij kampilobakter pri psih in mačkah v Iranu.

**Ključne besede:** psi; mačke; bakterije kampilobakter; zoonoza

# EFFECT OF THREE-DAY ACTH ADMINISTRATION ON CONCENTRATIONS OF CHOLESTEROL, CORTISOL, PROGESTERONE, TESTOSTERONE AND LH IN THE BOARS

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**Summary:** The objective of this study was to investigate effects of stress induced by ACTH on cholesterol, cortisol, progesterone, testosterone and LH concentrations in Swedish Landrace boars aged 6 to 7 months during ACTH administration and for 12 days after the cessation of treatment. ACTH treated boars (n=14) were given intravenously 10 mg/kg of ACTH for 3 days. Control group (n=14) received intramuscularly 1 mL of sterile 0.9% saline. The cortisol concentrations were significantly elevated ( $p < 0.0001$ , all) in all three days of ACTH treatment and day after the last ACTH injection ( $p < 0.01$ ) in treated boars. In twelve days after the cessation of treatment, cortisol levels remained on physiological levels. During all three ACTH treatment days and also one day and five days after the last ACTH dosage, cholesterol concentrations were significantly decreased ( $p < 0.05$  to  $p < 0.0001$ , respectively) in comparison with control boars. Progesterone and testosterone concentrations were significantly increased ( $p < 0.001$  to  $p < 0.0001$ , respectively) during all three days of ACTH treatment. After the treatment there was no significant difference in progesterone and testosterone levels between treated and control boars. ACTH administration had no influence on LH levels in treated boars. Significantly reduced cholesterol concentrations in the serum of boars exposed to three-day ACTH induced stress shows its increased biotransformation, which is confirmed by the results of this paper.

**Key words:** boars; ACTH; stress; cortisol; hormones; cholesterol

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## Introduction

Attempts to define stress through the patterns of specific physiological parameters frequently yielded controversial results. In addition to its effects on changes in the secretion of pituitary gland and adrenal gland hormones, stress also affects the testicular and ovarian hormones, which are important for the animal reproduction system. It may have negative effects in certain phases of animal development and breeding and may reduce the reproduction capacities (1).

Stressful situations such as temperature changes (2), transport (3), mixing of animals from different litters (4), and competition inside groups may affect the growth (5, 6), development of endocrine system (7, 8) and reproduction system functions in pigs (9, 10, 11). Chronic stress in females reduces the secretion of FSH and LH, and affects the absence of LH secretion at the expected time before ovulation (12) and reduction the number of gravid females (9). Acute stress situations cause an increase in progesterone concentrations in the blood of cows (13) and pigs (14), but they have no effect on LH concentrations (15). Chronic stress stimulated by increased temperatures in summer reduces hormone synthesis in ovaries and leads to lower blood concentrations of progesterone (16)

and estradiol, up to 50% compared to the winter period (17). However, although artificially stimulated ejaculation has been found to have stressful impact and to increase the cortisol concentrations, but testosterone concentrations were not changed in boars (18). In a normal ejaculation cycle in pigs including male and female contact, testosterone concentrations were increased (19).

Contemporary studies are applied stress induction by exogenous ACTH or cortisol. Acute stress stimulated by one-time ACTH application significantly increased the cortisol concentrations in 30 to 90 minutes after administration (14, 20, 21). ACTH administration during several days is used in simulations of chronic stress. Three-day stress induction by ACTH administration stimulated biochemical changes in boars during the treatment and in several-week period after its completion (22).

The objective of this study was to evaluate the effects of three-day injections of ACTH on reproduction system hormone concentrations. For that purpose, cortisol concentrations were determined as well progesterone, testosterone and LH concentrations in blood of boars before, during and after artificially stress induction by ACTH. Also, impact on concentration of cholesterol as the main precursor for steroid hormone synthesis was monitored.

## Materials and methods

### *Experimental animals*

Boars (Swedish Landrace, n=28) aged 6 to 7 months with average weight  $124.3 \pm 5.7$  kg were used in the experiment. The boars were housed in individual pens on farms. The stable temperature varied between 9 and 15 °C. Water was available *ad libitum*. The animals were given 3 kg of feed concentrate *per day* containing the following components: crude protein 16.99%, crude fat 5.39%, crude fiber 5.26%, starch 37.96%, sugar 3.02% and ash 5.65%.

### *Treatment*

The boars were randomly assigned to either control (n=14) or ACTH treated (n=14) group. Both groups of animals were handled using restraint with a snare in the procedures of administering saline and ACTH and also in the procedure of

blood collection. Accordingly, both groups were introduced to the same stress conditions. Also, in order to condition the animals to handling using restraint both experimental groups were intramuscularly administered 1 mL of sterile 0.9% at 10.00 a.m. on the day before the treatment. Furthermore, on each of the three treatment days (day 2, 3 and 4), ACTH/saline was administered at 10.00 a.m. The ACTH group of boars was injected 10 mg/kg body mass of ACTH (1-39, from porcine pituitary, 80 IU/mg, Sigma-Aldrich Co., USA) into ear vein. Control group of boars were administered 1 mL of sterile 0.9% saline intramuscularly on three consecutive days as a placebo.

### *Blood sampling*

All boars were frequently handled using restraint with a snare and habituated to blood collection procedure to be performed *via* jugular vein on the day before the experimental period and on each day of the experiment 90 min after drug administration (ACTH or saline). Also, blood samples were collected on days 1, 5, 8 and 12 after the treatment (day 5, 9, 12 and 16). Approximately 15 mL of blood was obtained using a sterile syringe (Becton Dickinson S.A., Spain). Blood samples were immediately transferred to tubes for serum samples (SST, DB Vacutainer<sup>®</sup>, Preanalytical Solutions Belliver Industrial Estate, UK). Blood samples were subsequently centrifuged at  $750 \times g$  for 10 min, separated and stored at -20 °C until the analysis.

### *Hormone analysis*

Serum cortisol was determined by radioimmunoassay serum using commercially available radioimmunoassay Coat-A-Count Kit (Diagnostic Products Corp., USA) according to the manufacturer's instructions. The antibodies used against cortisol had the following specificity: cortisol 100%; progesterone 0.02% and aldosterone 0.03%. Samples were quantified in two assays, with average intra- and interassay coefficients of variation of 7.5% and 12.0%, respectively. The detection limit of assay was 0.1 nmol/L.

Serum progesterone was measured by a solid phase radioimmunoassay Coat-A-Count Progesterone (Diagnostic Products Corp., USA), used according to the manufacturer's recommendations. The antibodies used against progesterone

had the following specificity: progesterone 100%; 5 $\alpha$ -pregnan-3,20-dione 9.0%; 5 $\alpha$  hydroxyprogesterone 3.4%; cortisol 0.03% and testosterone 0.1%. Samples were quantified in two assays, with average intra- and interassay coefficients of variation of 9.2% and 14.0%, respectively. The detection limit of assay was 0.1 nmol/L.

Serum testosterone levels were determined using a Coat-a-Count kit (Diagnostic Products Corp., USA) with a detection limit of 0.15 nmol/L. All samples were analyzed in a two assay with intra- and interassay coefficients of variation of 7.0% and 10.0%, respectively.

Serum LH was measured by homologous double antibody RIA using specific antiserum prepared in rabbits (23). Samples were quantified in two assays, with intra-assay coefficients of variation of 5.3 % and the detection limit of assay was 0.1  $\mu$ g/L.

### *Cholesterol analysis*

In serum samples all blood chemical measures were analyzed on a Technicon RA-1000 System Spectrophotometer (Technicon® Instruments Corporation, Tarrytown, New York). Blood serum cholesterol levels were measured by the enzymatic endpoint method using a commercial kit (Randox Laboratories Ltd.), yielding intra- and inter-assay coefficients of variation of 4.8% and 7.6%, respectively.

### *Statistical analysis*

The statistical analyses were performed using statistical package 6.1 Statistica® software (Stat-Soft® Inc., USA). The observations were analyzed by the analysis of variance. The statistical model included the effects of treatment, period, and interaction between period and treatment. Results were expressed as mean  $\pm$  SE. Differences in cholesterol and hormone concentration between control and ACTH groups of animals were analyzed by Student's *t*-test for independent pairs. The repeated measurements of cortisol, testosterone, progesterone and cholesterol concentration across the time were analyzed using a two-way repeated measures analysis of variance, with the Greenhouse-Geisser adjustment ( $\epsilon$  value) to the *p* values to account for any violation of the sphericity assumption. The repeated measures factor was time (day of checking)

and the between groups factor was ACTH administration. Probability values  $\leq 0.05$  were considered to be statistically significant.

## **Results**

### *Cholesterol*

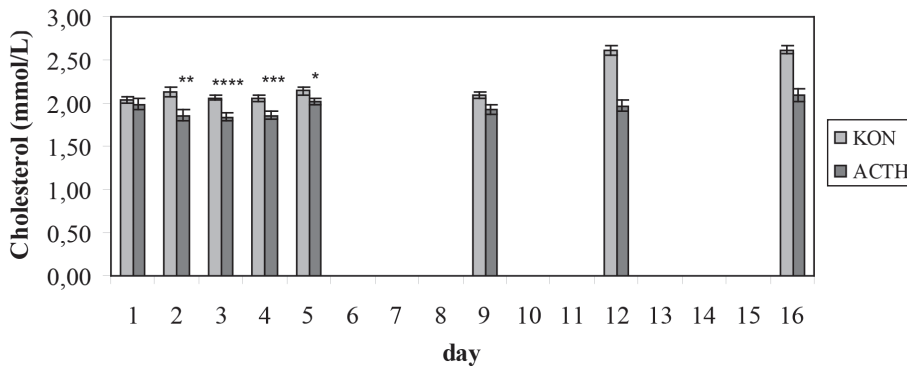
During all three ACTH treatment days, cholesterol concentrations were significantly decreased ( $p < 0.01$  to  $p < 0.0001$ , respectively) in treated boars (Fig. 1). The mean concentrations of cholesterol during treatment days were  $1.84 \pm 0.041$  to  $1.86 \pm 0.042$  mmol/L in ACTH group, whereas in control group they were  $2.06 \pm 0.03$  to  $2.13 \pm 0.06$  mmol/L. Also, one day (day 5) and five days (day 9) after the last ACTH dosage, concentrations of cholesterol were significantly lower ( $p < 0.05$ , respectively) in comparison with control animals. However, there were no significant differences in cholesterol concentrations during treatment in control and also in ACTH group by repeated analysis of variance.

### *Cortisol*

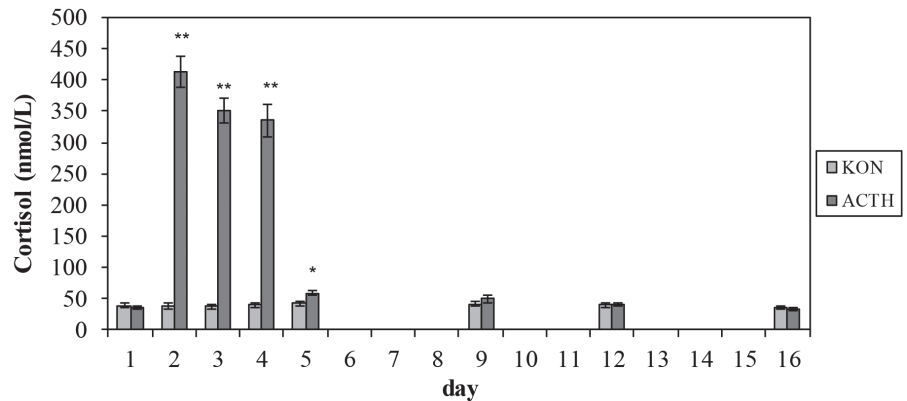
Cortisol concentrations were significantly elevated ( $p < 0.0001$ , all) in all three days of treatment in treated boars (Fig. 2). The mean concentrations of cortisol during treatment days were  $335.5 \pm 25.48$  to  $412.5 \pm 24.74$  nmol/L in ACTH group and  $36.9 \pm 3.49$  to  $39.2 \pm 3.06$  nmol/L in control group. One day after the last ACTH dosage (day 5), concentration of cortisol remained elevated ( $58.2 \pm 3.99$  nmol/L) and it was significantly higher ( $p < 0.01$ ) in comparison with control animals. There was no significant difference on days 9, 12 and 16 after the treatment. In control animals, the cortisol response remained stable throughout the experimental period. During treatment significant differences by repeated analysis of variance for cortisol concentrations in ACTH group were obtained ( $\epsilon = 0.404$ ,  $p < 0.001$ ).

### *Progesterone*

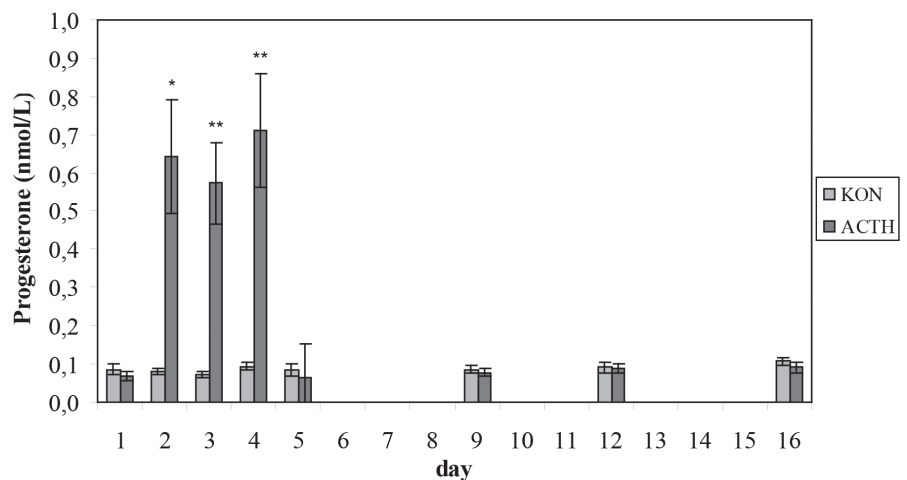
The progesterone pattern of ACTH boars was similar to cortisol pattern (Fig. 3). The concentrations were significantly increased ( $p < 0.001$  to  $p < 0.0001$ , respectively) during all three days of treatment in ACTH group of boars. The mean con-



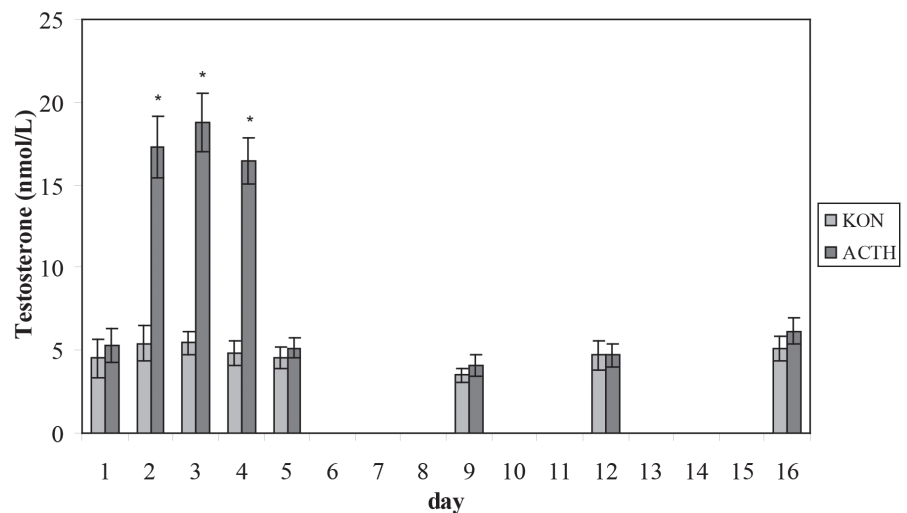
**Figure 1:** Serum concentrations of cholesterol (mmol/L; mean ± SE) during and after treatment with ACTH in ACTH (n = 14) and control group (KON; n = 14) of boars. Significant differences between groups: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$



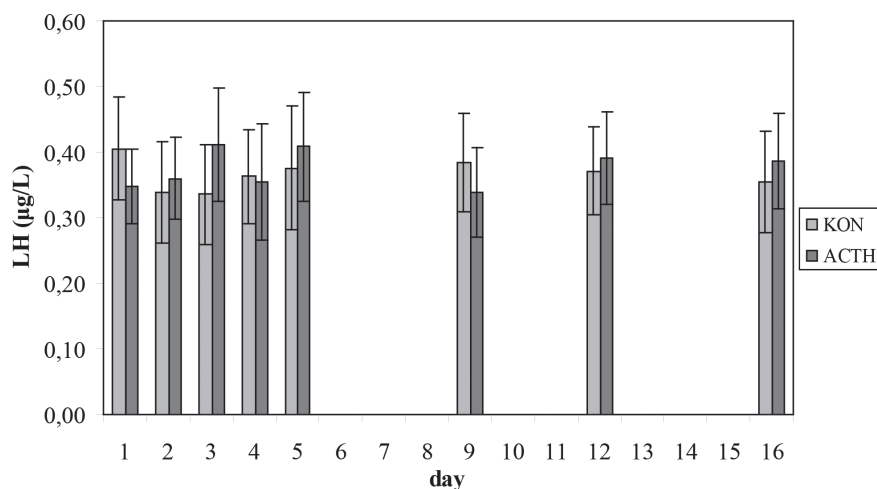
**Figure 2:** Serum concentrations of cortisol (nmol/L; mean ± SE) during and after treatment with ACTH in ACTH (n = 14) and control group (KON; n = 14) of boars. Significant differences between groups: \*  $p < 0.01$ , \*\*  $p < 0.0001$



**Figure 3:** Serum concentrations of progesterone (nmol/L; mean ± SE) during and after treatment with ACTH in ACTH (n = 14) and control group (KON; n = 14) of boars. Significant differences between groups: \*  $p < 0.001$ , \*\*  $p < 0.0001$



**Figure 4:** Serum concentrations of testosterone (nmol/L; mean ± SE) during and after treatment with ACTH in ACTH (n = 14) and control group (KON; n = 14) of boars. Significant differences between groups: \*  $p < 0.0001$



**Figure 5:** Serum concentrations of LH (mg/L; mean  $\pm$  SE) during and after treatment with ACTH in ACTH (n = 14) and control group (KON; n = 14) of boars

centrations of progesterone during treatment were  $0.57 \pm 0.106$  to  $0.71 \pm 0.149$  nmol/L in treated boars and  $0.072 \pm 0.009$  to  $0.094 \pm 0.008$  nmol/L in the control group. After the treatment there was no significant difference between the experimental groups of animals. Repeated analysis of variance shown significant differences for progesterone concentrations in group treated with ACTH ( $\epsilon = 0.338$ ,  $p < 0.0002$ ).

### Testosterone

Also, testosterone pattern of ACTH boars was similar to cortisol pattern during treatment, so the concentrations were significantly increased ( $p < 0.0001$ , all) in treated boars (Fig. 4). The mean concentrations of testosterone rose during the treatment from basal levels to the range of  $16.5 \pm 1.41$  to  $18.8 \pm 1.80$  nmol/L and stayed in the range  $4.5 \pm 1.15$  to  $5.5 \pm 0.69$  nmol/L in the control group. After the treatment there was no significant difference between treated and control boars. Repeated analysis of variance shown significant differences for testosterone levels in ACTH group during treatment ( $\epsilon = 0.604$ ,  $p < 0.001$ ).

### LH

There was no significant difference in LH concentrations between controls and treated boars during and after ACTH induction (Fig. 5). In both groups of boars mean concentrations were in the range  $0.34 \pm 0.076$  to  $0.41 \pm 0.086$  µg/L. During treatment repeated measures ANOVA showed no significant differences for LH concentrations in both experimental groups.

## Discussion

Various experimental models have been applied to explain the effects and consequences of stress on the animals. Different stressful conditions and their duration cause changes in cortisol secretion in pigs (2, 24). Adrenal gland response to ACTH stimulation depends on the intensity and duration of ACTH effects and there is a difference between the acute effects within a minute, and chronic effects which may take hours or days (25). Thus for instance, the levels of cortisol in pig serum is increased by low ambient temperature and reduced by high ambient temperature (2). Repetition of acute stressful situations reduced ACTH and cortisol levels, however at the same time the adrenalin level and heart rate remain increased (26). Chronic stress is changing the basic daily rhythm of ACTH and cortisol secretion in pig serum, which is characterized with an increase between 1.00 a.m. and 7.00 a.m. and fall of concentrations almost by half around 7.00 p.m. (27, 28, 29).

In this study, the application of three-day ACTH stress induction model reduced the individual differences in adrenal gland response in animals during the treatment. During three days of ACTH administration, a significant increase in serum cortisol concentration was observed 90 min after injection (from 34.7 to 415.5 nmol/L). This is consistent with previous reports in female pigs (7, 14, 30) and boars (22, 31). According to the previous results, cortisol concentration remains increased for 24 hours after the last administration of ACTH (22). High cortisol concentrations in serum of young boars were found in stressful situations such as territorial conflict (4) and sexual excitement (18).



This investigation monitored the effects of ACTH induced stress on steroid hormone concentrations and the main precursor of synthesis of steroid hormones of cholesterol. Endocytosis through cell membrane transported the cholesterol from blood into the cellular cytosol and the internal mitochondrial membrane (25). ACTH induces the transfer of cholesterol into the interior of adrenal gland cell mitochondrion, increasing the number of receptors for linking of LDL and HDL lipoproteins on cell membrane and providing the intake of lipoprotein through endocytosis into the cells (32). It also increases the activity of cholesterol esterase in lysosomes, and cholesterol esters from LDL or lipid drops are hydrolysed into free cholesterol. This increases the quantity of free intracellular cholesterol and reduces cholesterol concentration in circulation (25). Also, ACTH stimulates the desmolase activity, starting the process of steroidogenesis by side-chain cleavage on C<sub>21</sub> atom, where cholesterol is converted into pregnenolon (25, 33).

In this research, stress induction with ACTH reduced the cholesterol concentrations in boar serum during all three days of treatment. These results show a stimulated synthesis of cortisol and other steroid hormones, which is proven by the results of this study. Cholesterol concentrations in this experiment remained low in the week after ACTH induced stress, while steroid hormone concentrations back to control level. In acute stressful situations, such as temperature change, insignificant changes in cholesterol and cortisol levels were observed in the blood of pigs (2).

Also, in this study, stress induction with ACTH stimulated the synthesis of progesterone and testosterone in boars during ACTH administration. However, the applied stress induction model had no long-term effects on the concentrations of these hormones and by termination of ACTH administration, hormone concentrations returned to their pre-treatment levels. The observed concentrations of progesterone and testosterone in the blood of control group boars were in compliance with the reference values in previous studies (19, 34, 35). However, one-time ACTH administration caused an increase in progesterone concentrations in the serum of male guinea-pigs (36), pigs (14, 20, 37) and castrated boars (38). As in addition to its synthesis by ovaries, progesterone is also synthesized by the adrenal gland, it is deemed that progesterone may represent a significant factor in the inter-

pretation of hormone profile in the animal plasma during stress (14, 39, 40). Namely, in adrenalectomized pigs, there was no increased progesterone synthesis, which shows that progesterone secreted after the ACTH administration originates from the adrenal gland (39).

However, stressful situations with increased cortisol and progesterone also increased the testosterone concentrations in the blood of boars (19, 41) and horses (42). Also, administration of a single ACTH dose in rats (43), rabbits, guinea-pigs (36) and boars (41) at first stimulated the testosterone synthesis. However, several hours after ACTH administration, cortisol concentrations in blood were very high and testosterone concentrations were lower than normal physiological values (36, 41). Double effects of ACTH on testosterone level have not been explained. The assumption is that the initial testosterone secretion increase, found also in this study, is a result of ACTH effects on increased arterial blood flow in the adrenal gland or testicles (44). Also, ACTH administration had no effects on testosterone synthesis if the boars were adrenalectomised (45). The assumption is that the effects on testosterone synthesis are mediated by the adrenal gland, i.e. cortisol acts directly on testicles, by a mechanism independent from hypothalamus-pituitary gland control system (19, 41). Investigations in rats and guinea pig showed that glucocorticoids had a direct inhibition effect on the synthesis of testosterone in Leydig cells (36, 43, 46, 47).

Application of three-day ACTH stress induction model in this study had no changed of LH concentrations in the blood of boars. The results were in compliance with the previous studies of acute stress. Administration of single dose of ACTH in guinea-pigs (36), rats (43, 46) and pigs (12, 15) also had no changed LH serum concentrations. Measured LH concentrations in this research were similar to values determined in previous studies (21, 48). In chronic stress situations caused by ACTH administration during 7 or more days in pigs or rats, the secreted glucocorticoids caused a reduction of LH, FSH and testosterone concentrations in blood (39, 46, 49). It was found that a seven-day ACTH treatment inhibited the increase in basal LH concentration after ovariectomy in female pigs only when the adrenal gland was not removed (39).

In conclusion, in boars exposed to three-day ACTH stimulated stress significant reduction of cholesterol concentrations were observed which is

explained by its increased biotransformation and as result elevated serum concentration of cortisol and also progesterone and testosterone were measured.

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## VPLIV TRIDNEVNEGA DODAJANJA ACTH NA RAVEN HOLESTEROLA, KORTIZOLA, PROGESTERONA, TESTOSTERONA IN LH PRI MERJASCIH

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**Povzetek:** Z raziskavo smo želeli ugotoviti vpliv stresa, povzročenega z ACTH, na raven holesterola, kortizola, progesterona, testosterona in LH pri merjascih švedske deželne pasme, starih 6 do 7 mesecev, med samo aplikacijo ACTH in do 12 dni po prenehanju tretiranja. Živali v skupini ACTH ( $n = 14$ ) so 3 dni dobivale ACTH (10 mg / kg, i.v.), živali v kontrolni skupini ( $n = 14$ ) pa so prejele 1 ml sterilne fiziološke raztopine i.m. Koncentracija kortizola je bila pri tretiranih merjascih statistično značilno povišana ( $p < 0,0001$ ) v vseh treh dneh tretiranja in še naslednji dan po zadnji injekciji ACTH ( $p < 0,01$ ). Po 12 dneh je bila raven kortizola na fiziološki ravni. V primerjavi s kontrolnimi živalmi so imeli merjasci, tretirani z ACTH, značilno nižjo koncentracijo holesterola v vseh treh dneh tretiranja, pa tudi en dan in 5 dni po zadnjem odmerku ACTH ( $p < 0,05$  do  $p < 0,0001$ ). Koncentracije progesterona in testosterona so bile bistveno povečane ( $p < 0,001$  do  $p < 0,0001$ ) v vseh treh dneh tretiranja z ACTH, po končanem dodajanju pa ni bilo bistvenih razlik v koncentraciji progesterona in testosterona med tretiranimi in kontrolnimi merjasci. Tretiranje z ACTH ni vplivalo na raven LH. Občutno znižane koncentracije holesterola v serumu merjascev, ki so bili izpostavljeni 3-dnevnomu stresu, povzročenemu z aplikacijo ACTH, kaže na povečano biotransformacijo holesterola v steroidne hormone, kortizol, progesteron in testosteron, kar je v skladu z rezultati te raziskave.

**Ključne besede:** merjasci; ACTH; stres; kortizol; hormoni; holesterol

# EFFECT OF TREATMENT WITH TRIS-EDTA/CHLORHEXIDINE TOPICAL SOLUTION ON CANINE PSEUDOMONAS AERUGINOSA OTITIS EXTERNA WITH OR WITHOUT CONCOMITANT TREATMENT WITH ORAL FLUOROQUINOLONES

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**Summary:** *Pseudomonas aeruginosa* (*P. aeruginosa*) infection in ear canals of dogs is associated with severe otitis externa (OE). Traditional use of antibiotics alone or in combination with topical antiseptic solutions to treat *Pseudomonas* OE is often associated with the development of antibiotic resistance. Tris-EDTA/chlorhexidine solution is very active against several species of gram-positive and gram-negative bacteria *in-vitro*. Therefore, *in-vivo* effect of topical antiseptic solution containing Tris-EDTA/chlorhexidine 0.15% on clinical and microbiological parameters in 18 dogs diagnosed with *Pseudomonas*-associated OE, with (n=12) or without (n=6) the concomitant use of oral fluoroquinolones, was evaluated over an eight week period.

Dogs were evaluated on days 0, 7, 14, 21, 28, 42 and 56. Clinical signs: pain, oedema, erythema and stenosis of the ear canal were evaluated and scored from 0 to 4. Overall combined score including all clinical signs was also calculated to show all clinical signs as an independent variable. Ear canal swabs and scrubs were used to assess for culture and fluoroquinolone sensitivity, parasitological examination and cytological evaluation. Linear mixed effects model and the logistic regression with the random effect were used to evaluate the effect of Tris-EDTA/chlorhexidine (time) and fluoroquinolones treatment on clinical, cytological and bacteriological variables.

Results indicated that the number of rod shaped bacteria and neutrophil granulocytes decreased with time regardless of *fluoroquinolone* treatment ( $P < 0.05$ ). Similarly, all clinical signs were affected by Tris-EDTA/chlorhexidine treatment independently of fluoroquinolone treatment ( $P < 0.05$ ). Fluoroquinolone resistance was observed in *P. aeruginosa* in both, dogs that were, and those that were not treated with fluoroquinolones. Treatment with Tris-EDTA /chlorhexidine 0.15% combination solution seems to be beneficial for resolution of *Pseudomonas*-associated OE in dogs, which was independent of fluoroquinolone treatment.

**Key words:** otitis externa; dogs; *Pseudomonas aeruginosa*; Tris-EDTA; chlorhexidine; fluoroquinolones

## Introduction

*Pseudomonas aeruginosa* (*P. aeruginosa*) infection in an ear canal is usually associated with a severe otitis externa (OE) in dogs. Clinical features of *Pseudomonas* otitis include purulent discharge with severe erythema, ear canal erosions and ul-

cers with frequent bleeding, pain and discomfort. Tympanic membrane rupture can occur in cases assuming a chronic character, which is caused by proteolytic enzymes secreted by *P. aeruginosa*, and inflammatory cells' derived lizozymes. Such complications lead to otitis media (1,2).

Pathogenesis of canine otitis is complex and includes predisposing (narrow ear canals, high humidity, pendulous ear pinnae), primary (allergies, foreign bodies, ear mites and skin keratinisation

defects as most common), secondary (bacteria, yeasts) and perpetuating factors (hyperplasia of the ear canal tissue, ear drum or middle ear mucosa due to chronic inflammation) (1). Primary factors only are capable of causing OE. Other factors in immunologically competent animals are required to combine their virulence with each other to be able to cause OE. Hypersensitivity reaction is the most common primary cause of canine OE (> 40%) (1). Predisposing, primary, secondary as well as perpetuating factors should be recognized and eliminated for successful treatment of canine OE. *Pseudomonas* infection in OE is considered to be of secondary nature.

Treatment of *Pseudomonas* otitis includes topical and systemic antibiotics, and regular ear irrigation with antiseptic solutions. Treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids is recommended to control pain and inflammation (1). Treatments delivered topically are superior to systemic treatment regimens, because they achieve sufficient contact and drug therapeutic concentration (3, 4). Traditional use of antibiotics alone or in combination with antiseptic solutions to treat *Pseudomonas* OE is often associated with the development of antibiotic resistance, which increases the morbidity and the mortality of the disease, and predisposes the disease to assume chronic character (5, 6).

Tris-EDTA/chlorhexidine solution is very active against several species of gram-positive and gram-negative bacteria, and performs better than chlorhexidine solution alone (7, 8, 9). It also increases susceptibility of *P. aeruginosa* to enrofloxacin "in vitro" (10). Therefore, Tris-EDTA/chlorhexidine solution may prove to be beneficial for canine *Pseudomonas* OE patients (8, 9) and is unlikely to select for bacterial resistance (9).

The *in-vivo* activity of topical antiseptic solution containing Tris-EDTA/chlorhexidine against *Pseudomonas* OE in dogs has not yet been reported. Preliminary results of the recent multicenter double blind placebo controlled study reported good efficacy against purulent canine otitis. Specific species of bacteria involved were not reported (11). Therefore, the purpose of this study was to assess *in-vivo* effect of topical antiseptic solution containing Tris-EDTA/chlorhexidine 0.15% on clinical and microbiological parameters in dogs diagnosed with *Pseudomonas*- associated OE, with or without concomitant use of oral fluoroquinolones.

## Material and methods

Eighteen private-owned dogs diagnosed with OE associated with *P. aeruginosa* infection were included with the owners's consent. Dogs diagnosed with hypothyroidism or demodicosis were not included in the study, nor were patients included that were diagnosed with any of diseases that might require treatment unrelated to otitis. Dogs with hypersensitivity to products used to treat OE were also excluded. The study was approved by the Animal Ethical Committee, Ministry of Agriculture of Republic of Slovenia, No. 4.4.-27/2010.

All dogs were subjected to detailed clinical examination, including blood analysis, before being included into the study. A detailed case history was taken, with special emphasis on previous antibiotic treatment. Pain, oedema, erythema and stenosis of the ear canal were evaluated independently by at least two clinicians from the same clinical background and scored from 0 to 4 (11). The average score was then used for analysis.

Overall combined score including all clinical signs was also calculated to show all clinical signs as an independent variable.

After otoscopic evaluation of the ear canal, two sterile cotton swabs were inserted into the lumen and swabbed against the surface of the ear canal at the junction between the vertical and horizontal ear canals where the cartilage bends at about a 45° angle (12). The first cotton swab was used for the culture and sensitivity testing and the second was rolled onto one glass slide that was later heat-fixed and stained with a modified Wright's stain (Diff-Quik<sup>R</sup>, Median Diagnostics, Dudinggen, Switzerland).

Epidermal scrub was made for parasitological examination from the vertical ear canal using ear curette. Dogs have had then their ears thoroughly rinsed with water saline for removal of the inflammatory debris. Treatment with topical saline solution is beneficial even though it can cause some ear canal maceration (13). The ear canal was dried up using suction via the catheter.

All dogs included in the study were treated with Tris-EDTA /chlorhexidine 0.15% combination solution (OtodineR, Industria Chimica Fine ICF, Palazzo Pignano, Italy) once daily. Oral antibiotic (ciprofloxacin or enrofloxacin, 5 mg/kg SID) and/or oral methylprednisolone (1,0 mg/kg SID for 5-7 days, than 0,5 mg/kg every other day) treatments

were instituted by the attending practitioners/clinicians based on their personal clinical preferences. Similarly, the decision to discontinue such treatment was also made independently of those involved in the study; however, it could have been supported by laboratory reports provided by the study group.

Other ongoing treatment regimens for included dogs were standardized including regular rinsing of the ear canal (days 0, 7, 14, 21, 28, 42, 56) with saline solution for removal of the inflammatory debris (2). Owners of the dogs were instructed to fill the infected ear canals with Tris-EDTA /chlorhexidine 0.15% solution once daily accompanied by a gentle two minute massage of ears towards the head.

### *Parasitological and cytological examination*

Native smears of the epidermal scrubs were examined microscopically under low magnification (x40) for the presence of parasites. Cytological evaluation of the smears was performed in a semi-quantitatively manner (14), adjusted to an oil-immersion (x1000) magnification. Microscopic slides were screened at low (x100) magnification to locate most significant areas with cell monolayer. Inflammatory cells, rod shaped bacteria, cocci and yeasts were quantified in ten different oil-immersion fields (OIF, 1000x magnification). The average number was then calculated across all examined fields. Reference ranges were set and modified by the appropriate factor to adjust for 1000x magnification. Equal or more than 2 yeasts,  $\geq 10$  cocci,  $> 0$  rods and  $> 0$  inflammatory cells per OIF were considered abnormal (14).

### *Bacteriological examination*

Ear swabs were inoculated onto blood agar plates (Columbia agar supplemented with 5% sheep blood) and incubated aerobically at 37°C for 48+/-2 hours. After 24-h and 48-h incubation the plates were examined for the growth of *Pseudomonas spp.* or other pathogenic bacteria. Colonies morphologically consistent with *P. aeruginosa* were subcultured on fresh blood agar plates for subsequent identification.

Bacterial isolates were identified using methods described by Quinn and coworkers (15). The biochemical characteristics of isolates were determined using the commercial kit Api 20NE (BioMerieux, France).

### *Anti-microbial susceptibility testing*

The cultured bacteria were tested for the enrofloxacin and ciprofloxacin susceptibility using the disk-diffusion method by Kirby-Bauer according to accepted guidelines (Clinical and Laboratory Standards Institute M100-S20).

### *Statistical analysis*

The effect of treatment with antibiotic and time (Tris-EDTA /chlorhexidine 0.15% treatment) on the continuous outcomes of observed variables was verified with linear mixed effects model, where dogs were considered as a random effect. Interaction between treatment and time was also considered in the model. Variable methylprednisolone was included in the model as a controlling covariate in order to remove the possible confounding effect of this variable. The pre planned between-times and between-treatment comparisons were carried out with the contrast analysis.

The effect of time and treatment on binary outcomes was estimated with the logistic regression with the random effect. Like in the case of the continuous outcomes variable methylprednisolone was included as a controlling covariate to remove the confounding effect. The pre planned between-time and between-treatment differences were estimated with the contrast analysis. A p-value equal or less than 0.05 was considered as statistically significant. All analyses were performed with R language for statistical computing (version 2.8.1: R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.)

## **Results**

Eighteen dogs (mean age: 8.3 years, mean weight 21.77 kg) were included in this study. Breeds included were German Shepherd (5/18), Shar Pei (4/18), Cocker Spaniel (3/18), Highland Terrier (2/18), English Cocker Spaniel (1/18) and mixed-breed (3/18). Twelve dogs (12/18) were treated orally with antibiotics. Eleven dogs (11/18) were treated with methylprednisolone. All dogs had *P. aeruginosa* associated otitis externa at the beginning of the study. Atopic syndrome



was determined to be the primary cause for the disease in seventeen dogs (17/18). The primary cause for the disease could not be determined in one dog (1/18).

### Parasitology and cytology

No parasites were detected from ear canal epidermal scrubs from any of the patients.

All dogs (18/18) had rod shaped bacteria in their ear canal. Treatment with antibiotics had no effect ( $P=0.3$ ), whereas time had a significant effect on the reduction of rod shaped bacteria in the ear canal ( $P<0.0001$ ) (Table 1). Thirteen (13/18) dogs had rod shaped bacteria in their ear canals on day 56; from those eight (8/12) were and five (5/6) were not treated with oral fluoroquinolone.

Fourteen dogs (14/18) had cocci present in their ear canal at the beginning of the study. Treatment with antibiotics had no effect on the number of cocci in the ear canal ( $P=0.06$ ). The number of cocci did not change over time ( $P=0.08$ ) (Table 1). Six (6/18) dogs had cocci in their ear

canals on day 56; from those four (4/12) were and one (1/6) was not treated with oral fluoroquinolone.

Seven dogs (7/18) had yeasts present in their ear canal. Neither, treatment with antibiotics ( $P=0.4$ ) or time ( $P=0.4$ ) had an effect on the presence of yeasts in the ear canal (Table 1). Only one dog (1/18) had yeasts in the ear canal on day 56; the dog was not treated with fluoroquinolones. Treatment with methylprednisolone had no effect on the number of rod shaped bacteria, cocci or yeasts in the ear canal ( $P=0.3$ ).

*Neutrophil* granulocytes were detected in all dogs with *Pseudomonas* otitis (18/18). Their number was not affected by the treatment with antibiotic ( $P=0.6$ ); however, the number of *neutrophil* granulocytes reduced over time ( $P=0.0005$ ) (Table 2). Fourteen (14/18) dogs had *neutrophil* granulocytes in their ear canals on day 56; of those nine (9/12) were treated with oral fluoroquinolone and five (5/6) were not treated with oral fluoroquinolone.

Macrophages were detected in eight (8/18) cytology samples. Their number was not affected by the treatment with antibiotics ( $P=0.5$ ), nor it changed

**Table 1:** Ear cytology - microorganisms (No. (number) / OIF - 1000x magnification)

	Ear cytology (bacteria & yeast)						
	0	7	14	21	28	42	56
Rods*	(No./OIF)						
NoAtb	38.3±10.0	21.8±6.2	15.5±9.0	15.3±9.3	12.8±8.2	28.2±13.6	26.5±15.5
Atb	68.2±18.9	35.8±13.7	28.1±7.2	17.9±5.5	11.3±4.0	15.2±4.8	3.1±1.2
Cocci	(No./OIF)						
NoAtb	4.9±3.0	1.8±1.8	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	1.1±0.7
Atb	11.6±5.0	5.2±2.0	8.8±4.0	9.6±3.1	6.7±2.6	3.6±1.0	2.2±0.9
Yeast	(No./OIF)						
NoAtb	2.3±2.2	0.4±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1
Atb	0.2±0.1	0.1±0.1	0.1±0.1	0.5±0.5	0.1±0.1	0.0±0.0	0.0±0.0

Legend: Rods: rod shaped bacteria. Cocci: spherically shaped bacteria. NoAtb: not treated with antibiotics (n=6). Atb: treated with antibiotics (n=12). \* Significant effect of time -treatment with Tris-EDTA /chlorhexidine.

over time ( $P=0.4$ ) (Table 2). None of the animals had macrophages in their ear canal on day 56.

Ten (8/18) dogs had rod shaped bacteria present in their ear canals at the conclusion of the study (day 56), which were not determined as *P. aeruginosa* (*Proteus* sp., *E. coli* and *Corynebacterium* sp.) (2). Seven (7/12) were present in the fluoroquinolone treated patients and one (1/6)

was present in the patient not treated with fluoroquinolone. Treatment with antibiotics did not influence the presence of other rod shaped bacteria on day 56 of the study (OR=4.7; 95% CI: 0.68-4.0;  $P=0.1$ ), nor was the likelihood of the presence of other rods at the end of the study influenced by the treatment with methylprednisolone (OR=0.78; 95% CI: -2.1-1.7;  $P=0.9$ ).

**Table 2:** Ear cytology – inflammatory cells (No. (number) / OIF - 1000x magnification)

		Ear cytology (neutrophil granulocytes & macrophages)						
		Day						
		0	7	14	21	28	42	56
Neutrophil*	(No./OIF)							
NoAtb		1.4±0.7	0.7±0.1	0.3±0.1	0.6±0.2	0.4±0.1	0.6±0.1	0.8±0.4
Atb		1.2±0.3	0.9±0.2	0.6±0.1	0.4±0.1	0.4±0.1	0.6±0.1	0.2±0.1
Macrophage	(No./OIF)							
NoAtb		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Atb		0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Legend: NoAtb: not treated with antibiotics (n=6). Atb: treated with antibiotics (n=12). \* Significant effect of time -treatment with Tris-EDTA /chlorhexidine

**Table 3:** Ear and ear canal clinical signs score (0 (the variable (clinical sign) absent) – 4 (the variable (clinical sign) very evident))

		Clinical signs						
		Day						
		0	7	14	21	28	42	56
Erythema*	(Score)							
NoAtb		1.7±0.2	1.7±0.2	1.3±0.2	1.0±0.0	0.8±0.2	0.3±0.2	0.3±0.2
Atb		2.3±0.3	1.4±0.1	1.4±0.1	1.1±0.1	0.7±0.2	0.5±0.2	0.4±0.1
Oedema*	(Score)							
NoAtb		0.8±0.3	1.0±0.3	0.5±0.2	0.5±0.2	0.4±0.2	0.2±0.2	0.0±0.0
Atb		1.2±0.4	0.8±0.2	0.7±0.3	0.4±0.2	0.2±0.2	0.2±0.1	0.3±0.1
Pain* @	(Score)							
NoAtb		0.5±0.3	0.3±0.2	0.8±0.4	0.0±0.0	0.0±0.0	0.2±0.2	0.0±0.0
Atb		1.2±0.5	0.4±0.2	0.4±0.2	0.3±0.1	0.3±0.3	0.0±0.0	0.0±0.0
Stenosis*	(Score)							
NoAtb		1.2±0.4	0.8±0.4	0.8±0.4	0.3±0.2	0.4±0.2	0.2±0.2	0.0±0.0
Atb		1.8±0.4	1.2±0.3	0.8±0.3	0.4±0.2	0.3±0.2	0.2±0.1	0.2±0.1
ClinScore*	(Score)							
NoAtb		4.2±0.9	3.8±0.8	3.3±0.8	1.8±0.4	1.6±0.5	0.8±0.5	0.3±0.2
Atb		6.3±1.3	3.7±0.8	3.3±0.8	2.3±0.6	1.3±0.6	0.8±0.3	0.8±0.4

Legend: ClinScore: Overall clinical score combining Erythema, Oedema, Pain and Stenosis. NoAtb: not treated with antibiotics. Atb: treated with antibiotics. \* Significant effect of time -treatment with Tris-EDTA /chlorhexidine. @: significant effect of methylprednisolone on variable.

### Clinical signs

Treatment with antibiotics had no effect on ear canal erythema (P=0.6), oedema (P=0.9), stenosis (P=0.6) or pain (P=0.5). Similarly, treatment with methylprednisolone had no effect on erythema (P=0.3), oedema (P=0.6) and stenosis (P=0.3); however, it had a significant effect on pain (P=0.048).

Time had a significant effect on all clinical signs individually (P<0.001) (Table 3).

Clinical signs estimated together as one overall variable were also not affected by treatment with antibiotics (P=0.6), nor were they affected by the treatment with methylprednisolone (P=0.2). The severity of overall clinical signs diminished over time (P<0.0001) (Table 3).

### Bacterial culture

All dogs included in the study had their initial bacterial culture positive for *P. aeruginosa*. Six dogs that were treated with antibiotics (6/12) had their bacterial culture still positive for *P. aeruginosa* on day 56. One dog (1/6) that was not treated with antibiotics had its bacterial culture still positive for *P. aeruginosa* on day 56. Treatment with antibiotics had no influence on the bacterial cultures to test positive for *P. aeruginosa* at the conclusion of the study (OR=1.75; 95% CI: 0.2-14.4; P=0.6). Similarly, treatment with methylprednisolone had no influence on the bacterial cultures to test positive for *P. aeruginosa* at the conclusion of the study (OR=2.8; 95% CI: 0.3-21.7; P=0.3).

### Antibiotic resistance of *Pseudomonas sp.*

Six dogs (6/18) had resistant *Pseudomonas sp.* present in their ear canal on the initial bacteriology, which increased to twelve (12/18) on further evaluations. Treatment with antibiotics was not shown to increase the likelihood for *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones (OR=1.2; 95% CI: 0.016-4.7; P=0.3). Treatment with methylprednisolone also was not influencing *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones (OR=2.3; 95% CI: 0.5-1.7; P=0.1). Similarly, the development of specific antibiotic resistance against enrofloxacin or ciprofloxacin was not affected by treatment with fluoroquinolones or methylprednisolone (P>0.05).

### Discussion

This paper evaluated clinical, cytological and bacteriological characteristics of canine OE after topical treatment with Tris-EDTA /chlorhexidine 0.15% solution in cases where the disease was complicated with *P. aeruginosa* infection. Parameters were compared between cases that were and those that were not treated with fluoroquinolones. Significant observations were: 1) When dogs were treated with Tris-EDTA /chlorhexidine 0.15% combination solution, oral antibiotic treatment did not contribute to the resolution of *Pseudomonas* OE and 2) *P. aeruginosa* may be selected for antibiotic resistance regardless of the presence of the antibiotic treatment.

*Pseudomonas aeruginosa* infections are challenging to manage due to its potential multidrug resistance and the ability to produce several extracellular factors, which are involved in the expression of virulence (16). The unpredictable behaviour of *P. aeruginosa* with regards to antibiotic resistance was also observed in this study. Antibiotic resistance developed at some point of the study in most cases that were treated with antibiotics, as well as in cases that were not treated with antibiotics. Regardless of that effect, 11/18 dogs in our study were negative for *P. aeruginosa* on bacterial culture on day 56, which was not affected by the antibiotic treatment.

The dose of enrofloxacin at 5 mg/kg SID used in this study is lower than recently suggested (17). However, the dose of 20 mg/kg SID is often avoided due to high incidence of side effects and overwhelming costs (17).

Treatment with Tris-EDTA /chlorhexidine 0.15% combination solution was proven safe in dogs with OE (8). Similarly, no side effects were observed in this study. Tris-EDTA /chlorhexidine 0.15% solution was found "in-vitro" to be active against all the pathogens most commonly involved in canine otitis, including *P. aeruginosa*, which showed sensitivity at dilutions between 1:8 and 1:32 (9). Tris-EDTA also has the ability to potentiate the penetration of enrofloxacin into the bacterial cell by mechanisms involving magnesium cations, which effectively reduces the bacterias' minimum inhibitory concentrations (5, 18). Such beneficial effects were not appreciated in this study's population.

Clinical signs (pain, oedema, erythema and stenosis) were quantitatively scored and analysed separately, as well as unified under the cumulative score and analysed as an overall clinical variable. This was done to minimize the effect of individual clinician's clinical estimates and to minimize possible deviation in the animal presentation (stoic animals vs. easily excitable animals). The resolution of clinical signs was similar between dogs that were or were not treated with fluoroquinolones. Treatment with methylprednisolone was included in the statistical analysis to evaluate its potential effect on the outcome of all investigated parameters and should not be interpreted as a function that would show the specific effect on any particular clinical signs. Methylprednisolone has not been found to have any influence

on the disease outcome; however, it reduced pain in treated animals, as expected.

Cytological analysis of ear swab preparations is an important diagnostic tool to diagnose, estimate the severity of, and the success of implemented treatments for OE. It is more sensitive than bacterial culture at detecting organisms in the external ear canal (19). The presence of rod shaped bacteria, cocci, yeasts, *neutrophil* granulocytes and macrophages was quantitatively assessed. Epithelial cells were not included in the analysis, because of unpredictable presence in the clinical sample (14). Yeast and cocci infections resolved by the end of the study in all cases.

Rods are not part of the normal canine external ear canal flora; therefore, any presence of rods in the ear canal is considered pathological (20). Thirteen dogs (13/18) had rod shaped bacteria present in their ear canal on day 56 (Table 1). In cases where bacterial culture was negative to *P. aeruginosa*, other species of rod shaped bacteria (most frequently *Corynebacterium sp.*) were colonizing the ear canal. In cases where rod shaped bacteria were detected on the cytological smear, but no growth was present on the bacterial culture, the positive cytology result was attributed to the presence of dead bacteria in the cytological smear, or bacteria deteriorated during the process of sampling and/or culture procedures.

Inflammatory cells or phagocytosis should not be seen in cured ears' cytology smears (14), although some exceptions were reported (20). *Neutrophil* granulocytes were present on day 56 in 7/18 ear swabs. Nevertheless, their number was low and no phagocytosis was seen. The presence of neutrophil granulocytes in these cases could be attributed to unresolved underlying cause of OE. Our results indicate that the resolution of cytological indicators was not influenced by the treatment with fluoroquinolones, nor were they influenced by the treatment with methylprednisolone.

In conclusion, in this study the use of systemic fluoroquinolones had no beneficial effect on the outcome of *Pseudomonas* OE. Treatment with Tris-EDTA /chlorhexidine 0.15% combination solution seems to be efficient independently of antibiotic treatment, not disregarding the potential differences between topical and systemic antibiotic treatment. Treatment modalities without the use of antibiotics should be investigated to treat canine *Pseudomonas* OE.

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## UČINKOVITOST TOPIKALNEGA ZDRAVLJENJA PSEUDOMONASNEGA OTITISA Z RAZTOPINO TRIS-EDTA/KLORHEKSIDIN PRI PSIH Z VZPOREDNIM ZDRAVLJENJEM Z ORALNIMI FLUOROKINOLONI ALI BREZ LE-TEH

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**Povzetek:** Okužba zunanjih sluhovodov z bakterijo *Pseudomonas aeruginosa* (*P. aeruginosa*) povzroči hudo gnojno vnetje (otitis externa-OE). Uveljavljeno zdravljenje z antibiotiki, tudi v kombinaciji s topikalnimi antiseptičnimi raztopinami, pogosto vodi v razvoj rezistentnih sevov *P. aeruginosa*. Raztopina Tris-EDTA/klorheksidin dobro deluje proti mnogim vrstam gramsko pozitivnih in gramsko negativnih bakterij *in vitro*. V obdobju osmih tednov smo spremljali *in vivo* učinkovitost 0,15 % raztopine Tris-EDTA/klorheksidin na klinične in mikrobiološke parametre pri 18 psih s psevdomonasnim OE. Psi so bili razdeljeni v skupino, ki je oralno prejela fluorokinolone, in v skupino, ki fluorokinolonov ni prejela.

Meritve smo opravili na dan 0, 7, 14, 21, 28, 42 in 56. Klinične parametre vnetja (bolečina, otekline, rdečina in zožitev sluhovoda) smo ocenjevali na lestvici od 0 do 4. Analiziran je bil tudi skupen kliničen faktor, ki je upošteval kvantitativno oceno vseh kliničnih parametrov in jih na ta način prikazal kot neodvisno spremenljivko. Ostružke in brise zunanjih sluhovodov smo uporabili za citološko preiskavo, mikroskopsko parazitološko preiskavo, gojiščno preiskavo in antibiogram. Linearni fiksni učinek modela in logistična regresija z naključnim učinkom so bili uporabljeni za analizo delovanja Tris-EDTA/klorheksidina (čas) in fluorokinolonov na spremenljivke kliničnih, citoloških in bakterioloških parametrov.

Ugotovili smo postopno zmanjševanje števila paličastih bakterij in zmanjševanje števila nevtrofilnih granulocitov, neodvisno od zdravljenja s fluorokinoloni ( $P < 0.05$ ). Zdravljenje z raztopino Tris-EDTA/klorheksidin je vplivalo na klinične parametre neodvisno od zdravljenja s fluorokinoloni ( $P < 0.05$ ). Odpornost *P. aeruginosa* se je pojavila tako pri psih, ki so bili zdravljeni s fluorokinoloni kot pri tistih, ki z njimi niso bili zdravljeni. Raztopina Tris-EDTA/klorheksidin 0.15 % se je izkazala za učinkovito pri zdravljenju psevdomonasnega OE pri psih, neodvisno od zdravljenja s fluorokinoloni.

**Ključne besede:** otitis externa; psi; *Pseudomonas aeruginosa*; Tris-EDTA; klorheksidin; Ciprofloksacin; enrofloksacin

# ANALYSIS OF CONFORMATION TRAITS OF THE POSAVJE HORSE IN SLOVENIA

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**Summary:** Posavje horse is an autochthonous horse breed in Slovenia as well as in Croatia. Slovenian population of Posavje horse is traditionally reared in the lower flow of the river Sava, in the surroundings of Krško and Brežice. Breeding program for the Posavje horse was accepted in the year 2005 when the Slovenian Association of Posavje Horse Breeders was established, too. The aim of the study was to analyse the conformation and gait traits of the Slovenian population of Posavje horse. Likewise, we tried to evaluate the most known fixed effects, affecting the included traits. Data were collected during the classifications of Posavje horse, performed in the years 2000 to 2011 and evaluated by only one expert. In this study 557 horses were included, among them 44 stallions and 513 mares, born during the years 1999 to 2007. The scoring system included 9 measured and 11 scored traits. Data were analysed by GLM procedure of statistical package SAS/STAT considering sex, age at scoring and birth year as fixed effects. Posavje horses had in average  $141.9 \pm 3.48$  cm height at withers (stick) and  $144.5 \pm 3.76$  cm croup height. Body length ( $152.8 \pm 5.28$  cm) was in average larger than the height at wither, thus indicating the rectangular body frame. The analysed Posavje horse population in Slovenia included horses with bay (79%), dark bay (5%), chestnut (11%), grey (3%) and black (2%) coat colour. According to the literature Croatian population had very similar measured traits to the Slovenian population of Posavje horse. Considering LSM values of all included conformation traits, stallions had larger measurements and higher scores than mares what could be explained with sexual dimorphism.

**Key words:** Posavje horse; conformation traits; body measurements; gaits

## Introduction

Posavje horse is an autochthonous horse breed in Slovenia as well as in Croatia. Slovenian population of Posavje horse is traditionally reared in the lower flow of the river Sava, mostly in Krško and Brežice surroundings. The original breeding area of the Posavje horse is not closed, it is widespread also throughout Slovenia. In the year 2010 it was estimated that breeders in Slovenia reared around 1050 Posavje horses, where 545 were breeding mares and 96 were breeding stallions (Veterinary Faculty, Institute for breeding and

equine health). The breed was formed in the past on the base of the local mares in the Sava river basin, improved with cold-blooded stallions of the Belgian horse type. However, the preservation of the breed started in 1993, when the official Stud book for Posavje horse was established. Breeding program (1) was accepted in the year 2005 when the Slovenian Association of Posavje Horse Breeders was established, too. The Posavje horse is a small body framed horse with a smaller, thinner head, with straight profile and a moderately long neck. The body is compact due to the short back, and short but wide croup. The Posavje horse has a strong foundation, as well as large and tough hooves. The horse's legs are muscular, protected by additional fur. The horse is known for its calm

temperament and good conversion even in poor rearing conditions (2).

The aim of the study was to analyse conformation traits in the Slovenian population of the Posavje horse. Likewise, we tried to evaluate the most known fixed effects which affected the analysed traits.

## Material and methods

Data were collected during the Posavje horse classifications, taken after the horses achieved sexual maturity. Classification was performed for males and females prior to records in the Posavje horse Stud book, starting in the year 2000 when the first Posavje horses were classified in Slovenia. Lately, till 2011, the number of recorded horses differed by years as shown in Figure 1. Likewise, the number of horses per birth year which were recorded in the Stud book differed in years.

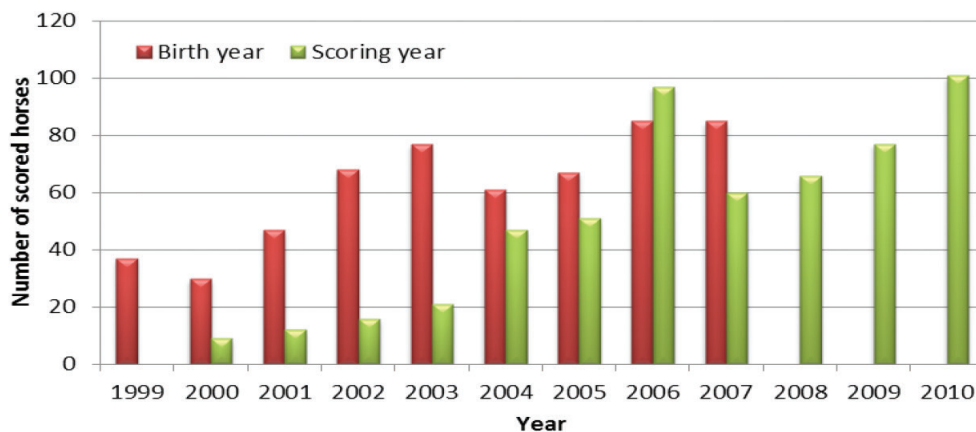
All included classifications of Posavje horse from 2000 to 2011 were performed by only one expert. The scoring system included 9 measured and 11 subjectively scored (1 to 10 point scale) traits, as well as coat colour description.

where 44 stallions and 513 mares were born during the years 1999 to 2007. Likewise, four body indexes were computed from the following measured data: cannon bone circumference/ height at wither (ICBC), chest width/ height at wither (ICW), chest depth/ height at wither (ICD) and croup width/ height at wither (ICrW).

Data were analysed by GLM procedure of statistical package SAS/STAT (3) considering sex, age at scoring and birth year as fixed effects (Model 1). Horses were divided into two groups by the age at scoring day, from 30 to 42 months, and from 43 to 60 months of age.

## Results

The height at wither measured by stick, and croup height in Posavje stallions was  $142.8 \pm 0.56$  cm and  $145.8 \pm 0.86$  cm while in Posavje mares  $142.0 \pm 0.24$  cm and  $144.8 \pm 0.27$  cm, respectively (Table 1). Body length (stallions:  $153.0 \pm 1.24$  cm, mares:  $153.3 \pm 0.39$  cm) was in average larger than height at wither, thus indicating a rectangular body frame. Stallions and mares were wider in croup ( $56.8 \pm 0.67$  cm,  $56.1 \pm 0.21$  cm) compared



**Figure 1:** Number of scored horses by birth year and scoring year

Database included data of measured and scored traits of totally 712 Posavje horses (137 stallions, 575 mares) which were classified before they were recorded in the Stud book. This study, however, includes only data of Posavje horses, aged 30 to 60 months in the scoring day. Data of younger and older horses as well as outliers were excluded from further analyses. Data of conformation traits used in this study belong to 557 horses of Slovenian Posavje horse population,

$$y_{ijk} = \mu + S_i + A_j + Y_k + e_{ijk} \quad \text{Model 1}$$

Where:

$y_{ijk}$  – conformation trait

$S_i$  – sex;  $i = 1, 2$

$A_j$  – age at scoring day;  $j = 1, 2$

$Y_k$  – birth year;  $k = 1, \dots, 11$

$e_{ijk}$  – residual

to chest ( $49.5 \pm 0.80$  cm,  $44.6 \pm 0.25$  cm). The measured cannon bone circumference was  $23.0 \pm 0.16$  cm (stallions) and  $20.9 \pm 0.07$  cm (mares). Among ten scored traits with the predicted scale from 1 to 10, there was no trait that scored the entire scale. Most traits (breed type, head, front part, middle part) were scored from 5 to 9, while two traits (rear legs, gaits correctness) from 5 to 8. Similarly, the neck and rear part scored from 6 to 9. Front legs were scored with the lowest scores from 4 to 8, while gait efficiency from 6 to 10.

Significant differences between stallions and mares of the Posavje horse were recorded at

three measured, six scored and two body indexes (Table 1). Stallions were higher in wither (tape measured) (156.92 cm), had larger cannon bone circumference (23.02 cm) and larger chest width (49.48 cm) than mares (153.70 cm, 20.94 cm, 44.61 cm), respectively.

Significant differences between stallions and mares existed also in scored traits. Stallions expressed breed type significantly better (7.42) than mares (7.02). Also head, middle part of the body and rear legs were significantly better scored in stallions (7.37, 7.28, 7.03) compared to mares (6.86, 7.02, 6.43). Gait correctness in stallions

**Table 1:** Least square means (LSM), standard errors (SE) and p-values for the effect of sex

	Stallions			Mares			p-values
	n	LSM	SE	n	LSM	SE	
<b>Measured traits (cm)</b>							
Height at wither – stick (WH)	44	142.8	0.56	512	142.0	0.24	ns
Height at wither – tape	44	156.9	0.70	510	153.8	0.30	0.001
Chest girth	44	188.2	1.57	511	187.0	0.67	ns
Cannon bone circumference (CBC)	44	23.0	0.16	494	20.9	0.07	< 0.001
Chest depth (CD)	21	67.7	0.67	510	67.6	0.21	ns
Croup height	21	145.8	0.86	510	144.8	0.27	ns
Chest width (CW)	21	49.5	0.80	510	44.6	0.25	< 0.001
Croup width(CrW)	20	56.8	0.67	510	56.1	0.21	ns
Body length	20	153.0	1.24	505	153.3	0.39	ns
<b>Scored traits (1-10)</b>							
Breed type	44	7.4	0.11	512	7.0	0.05	0.002
Head	40	7.4	0.12	512	6.9	0.05	0.001
Neck	40	7.5	0.12	512	7.3	0.05	0.050
Front part	40	7.7	0.11	512	7.5	0.05	ns
Middle part	40	7.3	0.11	512	7.0	0.05	0.043
Rear part	40	7.6	0.10	512	7.5	0.04	ns
Front legs	40	6.6	0.12	512	6.4	0.05	ns
Rear legs	40	7.0	0.10	512	6.4	0.04	< 0.001
Gait correctness	40	6.7	0.10	508	6.5	0.04	0.049
Gait efficiency	40	7.1	0.10	505	7.1	0.04	ns
Total score of scored traits	40	72.3	0.56	505	69.6	0.23	< 0.001
<b>Body indexes (%)</b>							
ICBC = (CBC/WH)*100	44	16.1	0.10	493	14.8	0.04	< 0.001
ICW = (CW/WH) *100	21	34.7	0.55	510	31.4	0.17	< 0.001
ICD = (CD/WH) *100	21	47.5	0.41	510	47.5	0.13	ns
ICrW = (CrW/WH) *100	20	39.9	0.45	510	39.5	0.14	ns

LSM – least square means, SE – standard errors



(6.70) was significantly better expressed than in mares (6.47), while gait efficiency was better expressed in mares, but the difference between sexes was not significant. The differences between sexes were significant in the two (ICBC, ICW) of four body indexes. ICBC and ICW were higher in stallions ( $16.1 \pm 0.10\%$  and  $34.7 \pm 0.55\%$ ) compared to mares ( $14.8 \pm 0.04\%$  and  $31.4 \pm 0.17\%$ ). Scored traits had higher scores at stallions with the exception of gait efficiency where a more intensive selection is seen in stallions compared to mares.

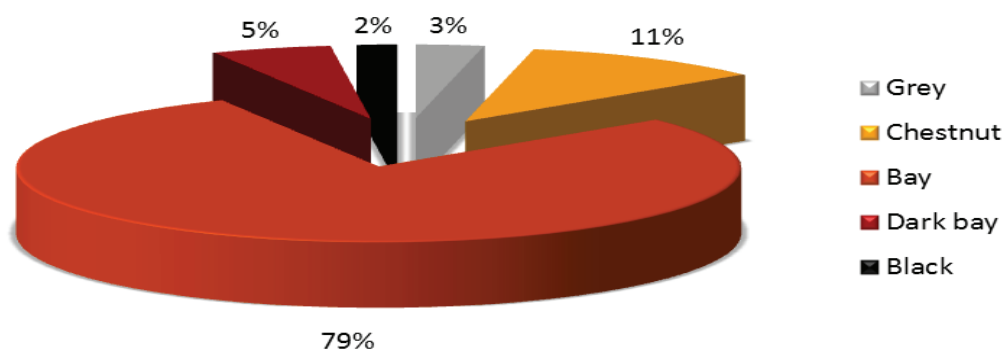
Differences in conformation traits between younger (30 - 42 months) and older (43 - 60 months) groups of horses were not significant with the exception of body length (Table 2). On

the other side, differences in conformation traits among birth years were significant for all scored traits, all body indexes and for almost all measured traits. The effect of birth year was not significant for height at wither (tape and stick measured), croup height and body length. Within the effect of birth year the sire effect could be expressed, because each sire did not have offspring in all studied years (Table 2).

In the time of conformation traits scoring the coat colour description was performed, too. The Posavje horse population in Slovenia included horses with bay (79%), dark bay (5%), chestnut (11%), grey (3%) and black (2%) coat colour (Figure 2).

**Table 2:** p-values for the effects of age at scoring and birth year

	Age (p-values)	Birth year (p-values)
<b>Measured traits (cm)</b>		
Height at wither – stick (WH)	ns	ns
Height at wither – tape	ns	ns
Chest girth	ns	0.001
Cannon bone circumference (CBC)	ns	0.002
Chest depth (CD)	ns	< 0.001
Croup height	ns	ns
Chest width (CW)	ns	< 0.001
Croup width (CrW)	ns	0.001
Body length	0.021	ns
<b>Scored traits (1 - 10)</b>		
Breed type	ns	< 0.001
Head	ns	< 0.001
Neck	ns	< 0.001
Front part	ns	< 0.001
Middle part	ns	< 0.001
Rear part	ns	< 0.001
Front legs	ns	< 0.001
Rear legs	ns	0.041
Gait correctness	ns	0.025
Gait efficiency	ns	< 0.001
Total score of scored traits	ns	< 0.001
<b>Body indexes (%)</b>		
ICBC = (CBC/WH)*100	ns	< 0.001
ICW = (CW/WH) *100	ns	< 0.001
ICD = (CD/WH) *100	ns	< 0.001
ICrW = (CrW/WH) *100	ns	< 0.001



**Figure 2:** Coat colour distribution in the Slovenian population of Posavje horse

## Discussion

The average values for measured traits especially height at wither measured by stick ( $141.9 \pm 3.48$  cm) has shown that the Posavje horse is a draft horse with small body frame compared to the well-known Noriker draft horse (156 – 162 cm) (4). However, LSM values of height at wither measured with stick were not in accordance with a breeding goal of the Posavje horse, which assumed 148 cm (145 – 152 cm) for stallions and 143 cm (135 – 148 cm) for mares (1). Slovenian population had very similar values of measured traits (Table 3) to Croatian population of the Posavje horse (5). LSM values of all included conformation traits have shown that stallions had larger measurements and higher scores than mares what could be explained with sexual dimorphism. Well expressed sexual dimorphism was observed also in the population of the Old Kladrub horse, a warm-blooded robust carriage horse, originally used for ceremonial purposes

by the Habsburg emperors (6). In the Posavje horse the croup height was higher than height at wither in average for 2.6 cm in the Slovenian and 2.7 cm in the Croatian population. This is considered as a well-known breed characteristic.

A large difference between populations existed only in body length, which could be the consequence of a different definition on how the trait is measured. Relatively low and similar standard deviations (Table 3) were seen at both populations which shows that Slovenian and Croatian population of Posavje horse are quite uniform. The exception existed just in chest width and body length where standard deviations were higher in Croatian, compare to Slovenian population. Similar chest girth measures ( $187.89 \pm 0.67$  cm) were found in Murgese stallions (7) compared to Posavje stallions ( $188.2 \pm 1.57$  cm) and cannon bone circumference in Murgese mares ( $21.07 \pm 0.07$  cm) compared to Posavje mares ( $20.9 \pm 0.07$  cm).

**Table 3:** Comparison between measured traits in Slovenian and Croatian population of the Posavje horse

Measured traits (cm)	Slovenian population (n = 557)		Croatian population (n = 107) (5)	
	Mean	SD	Mean	SD
Height at wither- stick	141.9	3.48	142.6	4.76
Height at wither – tape	153.9	4.39	154.2	5.15
Chest girth	187.3	9.93	191.2	9.10
Cannon bone circumference	21.0	1.15	20.9	1.00
Chest depth	67.2	3.11	71.2	2.86
Croup height	144.5	3.76	145.3	3.79
Chest width	44.9	3.88	49.7	5.75
Croup width	56.1	2.91	53.2	2.89
Body length	152.8	5.28	171.0	6.33

cm). Murgesse horse from the south of Italy is today a light draft horse used to saddles and harnesses while in the past it was bred as a draft horse and used to produce strong mules.

The scored traits in this study were scored on the scale only from 1 to 10 points. The explanation what exactly each point meant is very subjective, while 1 means that the trait was the worst expressed, and 10 that the trait was the most expressed in agreement with breeding goals. The average value for each trait means also the average value of the population, regarding to breeding goals in the breeding program (1). As was already mentioned, classifications were performed for all horses before they were recorded in the Stud book. However, foals or yearlings were also pre-selected by the owners either for further breeding or for slaughter, which is the reason that scored traits had higher average values than those presented by all horses in the complete population (also horses which have not been recorded in the Stud book). However, standard errors (Table 1) of scored traits were low. Likewise, the scores of all scored traits were represented by the values between 4 and 9 due to the lack of experience with the linear scoring system. Similarly, just 5 to 9 scores were used on the scale 1 to 10 for morpho-functional traits of the Andalusian horse (8). This means that most of the traits were scored with 5, 6 or 7 points, not covering the whole scale. However, Posavje horse scores showed heavier head ( $6.9 \pm 0.77$ ) and longer neck ( $7.3 \pm 0.74$ ) compared to Haflinger horse ( $4.73 \pm 1.37$ ;  $5.13 \pm 1.25$ ) (9).

Body indexes showed interdependence among measured traits, compliance of the body and coherence with the breed standards (10). On the base of body index (chest depth/height at wither) we could determine in which group, oriental (hot-blooded) (45.0 – 46.5 %), half-blooded (warm-blooded) (46.5 – 48.5 %), or cold-blooded (> 50.0 %), a horse or a population belongs to (10). Consequently, the Slovenian population of Posavje horse belongs to half-blooded (warm-blooded) group with the index chest depth/height at wither of stallions  $47.5 \pm 0.41\%$  and mares  $47.5 \pm 0.13\%$ .

The effect of age was not significant in almost all conformation traits what could confirm the fact that Posavje horse is an early matured breed (10). On the other hand, it was found that the effect of age significantly affected linear type traits of the Old Kladrub horse (11). Height at wither, neck and cannon bone circumference increased with age classes.

The proportions of different coat colours of Posavje horse in Slovenia (Figure 2) were similar to those in the studied population in Croatia (5) where 67% were bay, 17% dark bay, 2% chestnut, 3% grey and 11% black horses. In the Posavje horse population in Slovenia there were 12% more bay and 12 % less dark bay horses. In Slovenia, there were 9% more Posavje horses with chestnut coat colour and 9% less Posavje horses with black coat colour compared to Croatian population. The proportions of grey Posavje horses were equal (3%) in Slovenia and Croatia. However, breeding program for Posavje horse in Slovenia considers black, chestnut and grey coat colour as undesired in Posavje horse (1).

## Conclusions

Analysis of conformation traits in Slovenian population of the Posavje horse was the first step prior to the estimation of phenotypic and genetic parameters of measured and scored traits, as well as body indexes. In the Slovenian population of Posavje horse similar values of conformation traits were measured as in Croatian population. Considered similarity will be necessary in the future to standardize breeding programs (goals) in both populations of Posavje horse. However, the definitions of conformation traits will have to be harmonised to prevent mistakes, too.

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## ANALIZA ZNAČILNOSTI ZUNANJOSTI POSAVSKEGA KONJA V SLOVENIJI

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**Povzetek:** Posavski konj ali posavec je avtohtona pasma konj tako v Sloveniji kot na Hrvaškem. V Sloveniji tradicionalno redijo posavske konje ob dolnjem toku reke Save, še posebno v okolici Krškega in Brežic. Rejski program za posavskega konja je bil sprejet v letu 2005, ko je bilo ustanovljeno tudi Slovensko združenje rejcev konj pasme posavec. Namen raziskave je bil analizirati značilnosti zunanosti v slovenski populaciji posavskega konja. Prav tako smo skušali oceniti najbolj očitne sistematske vplive za vključene značilnosti. Podatki so bili zbrani med ocenjevanji ob sprejemih živali v rodovniško knjigo. Vsa ocenjevanja je opravil en ocenjevalec v letih od 2000 do 2011. V raziskavo je bilo vključeno 557 konj, od katerih je bilo 44 žrebcev in 513 kobil, rojenih med leti 1999 do 2007. Sistem ocenjevanja zunanosti je zajemal 9 merjenih in 11 subjektivno ocenjenih značilnosti. Podatki so bili analizirani s pomočjo analize GLM v statističnem paketu SAS / STAT. V model smo vključili spol, starost ob ocenjevanju in leto rojstva. Pri posavskih konjih je bila izmerjena povprečna višina v vihru (palica)  $141,9 \pm 3,48$  cm in višina v križu  $144,5 \pm 3,76$  cm. Dolžina telesa ( $152,8 \pm 5,28$  cm) je bila večja od višine vihra, kar kaže na pravokoten okvir. Slovenska populacija posavskega konja je vključevala konje v rjavi (79%), temno rjavi (5%), kostanjevi (11%), sivi (3%) in črni (2%) barvi. Pri slovenski populaciji so bile vrednosti merjenih značilnosti zelo podobne kot v hrvaški populaciji posavskega konja. LSM vrednosti značilnosti zunanosti so pokazale, da imajo žrebci bolj izražene zunanje značilnosti od kobil, kar je mogoče pojasniti s spolnim dimorfizmom.

**Ključne besede:** posavski konj; značilnosti zunanosti; telesne mere; hodi

# OCCURRENCE AND EFFECTS OF THE BIVALVE-INHABITING HYDROID *EUGYMNANTHEA INQUILINA* IN CULTURED MEDITERRANEAN MUSSELS (*MYTILUS GALLOPROVINCIALIS*) IN SLOVENIA

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**Summary:** The Mediterranean mussel (*Mytilus galloprovincialis*) is the most common bivalve species bred in the Slovenian sea. The mussels are bred on three locations, i.e., Seča, Strunjan and Debeli rtič in natural fisheries–shellfish farms. From November 2007 to October 2008, 960 adult cultured Mediterranean mussels were randomly sampled and included in our study. Water temperature, oxygenation and salinity were measured at each sampling. The mussels were measured and weighted to calculate the condition index and microscopically examined for the presence of hydroids. No increased mortality occurred during the sampling. Hydroids of *Eugymnanthea inquilina* were detected with 60.6% prevalence, mostly during summer. They were either attached to the mantle epithelium or found inside the mantle cavity. No alterations were observed at the point of attachment. The differences in the prevalence of infection in different seasons were statistically significant. Oxygen content and salinity had a statistically significant impact on the presence of infection, while temperature did not. The average condition index of mussels inhabited by *E. inquilina* was significantly lower than in uninfected mussels, indicating a parasitic relationship between *Mytilus galloprovincialis* and *Eugymnanthea inquilina*.

This is the first report on the occurrence of *E. inquilina* in Mediterranean mussels from the Slovenian sea.

**Key words:** *Eugymnanthea inquilina*, *Mytilus galloprovincialis*, histopathology, condition index, Gulf of Trieste

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## Introduction

The Slovenian sea, part of the Gulf of Trieste, the northernmost end of The Adriatic Sea, represents a specific and rich habitat, highly influenced by considerable fluctuations of temperature, oxygenation and salinity. The Mediterranean mussel (*Mytilus galloprovincialis*) is one of the most common bivalve species in the Slovenian sea and the most often bred bivalve. The mussels are bred on three locations, i.e., Seča, Strunjan and Debeli rtič in natural fisheries–shellfish farms.

The farms are quite small and composed of 5 fields, each measuring 20 000 m<sup>2</sup>. The annual production of each field is from 25 to 30 tons. The high density of mussels in aquacultures can lead to the spread of several epizootic diseases, which can affect the revenues and farming production. One of the endobionts found in Mediterranean mussels is the hydroid *Eugymnanthea inquilina* (Cnidaria, Hydrozoa). It has so far been reported in Greece (1, 2), Italy (3), Croatia (4) and Japan (5), but no record of this species exists in Slovenia. The athenate solitary hydroid stage of *E. inquilina* attaches itself with a basal disc in the mantle cavity of *M. galloprovincialis* and other bivalves (3). It is unclear what, if any, are the effects of this organisms on the survival and condition of the host

organism. Kubota (6) hypothesises that the relationship between the host and endobiont is commensalism with no harmful or beneficial effects to either organism. On the other hand, Rayyan et al. (2) and Galinou-Mitsoudi et al. (7) conclude that a parasitic relationship is more likely, since the presence of hydroids was correlated with lower growth rates and condition indices of the mussels. On the other hand, Mladineo et al. (8) found no deteriorating impact on host reproduction or condition index, but did observe ultrastructural alterations (cell desquamation) in host cells at the attachment site. The possibility of mutualism between *E. inquilina* and *M. galloprovincialis* has also been proposed; Piraino et al. (3) found an indication that the hydroids may have a protective role against mussel parasites.

In order to confirm the presence of *Eugymnanthea inquilina* in Mediterranean mussels from the Slovenian sea, we collected and histologically examined mussels from two shellfish farms. We measured the mussel condition index in order to ascertain potential effects of the hydroid on the condition of the farmed mussels. To observe any effects of environmental conditions on the presence and abundance of the *E. inquilina* polyps, we also measured water temperature, salinity and oxygenation. The prevalence of *E. inquilina* was correlated to the presence of intracellular ciliates in the same samples (9) in order to ascertain whether the presence of *E. inquilina* has any effect on the prevalence of other endobionts in the Mediterranean mussel. This is the first report on the occurrence of this organism in Slovenia.

## Materials and methods

80 adult cultured Mediterranean mussels (*Mytilus galloprovincialis*) from shellfish farms in Seča and Strunjan were stripped directly from ropes at a depth of approximately 3 metres monthly from November 2007 to October 2008. In total, 960 adult Mediterranean mussels were collected and included in our study. Water temperature, oxygenation and salinity were measured at each sampling at the depth of sampling (3 m). Oxygenation measurements were not performed in March. Water temperature and oxygenation were measured using a thermometer "MultiLine P4 – Oxi 320 Set" with a dissolved oxygen probe "Cellox 325" (WTW). Water salinity was measured using a hand-held

refract meter "S/Mill-E. S= 0-100‰" (ATAGO).

Live adult mussels were transported to the laboratory within one hour in a classic cooling bag. Organisms attached to the shell were carefully removed and the shells were washed with fresh water.

The length of the mussels was measured from the hinge to the longest part of the shell. The shell was opened and excess water was removed. The total weight of each mussel was measured and the flesh was carefully removed, drained on double absorbent paper and weighed. The total weight and weight of the flesh were determined to  $\pm 0.01$ g with an electronic balance. The flesh condition index was calculated by means of the formula "condition index = fresh flesh weight  $\times$  100/total weight".

A standard section through the visceral mass was performed after weighing. Samples were immediately placed in 10% formalin solution at room temperature for no more than 24 hours and were routinely paraffin embedded. Four- $\mu$ m-thick sections were stained with haematoxylin and eosin (HE) and one slide per mussel was examined under a light microscope for the presence of hydroids.

Morphometric analyses were performed on tissue slide photographs using a digital camera and Microphot microscope. Measurements of hydroids were performed using the computer programme NIS-Elements BR. The length of fifty randomly chosen hydroids was measured and the average value of the measured parameters was calculated.

The statistical calculations were performed using the SPSS software package. The independent samples t-test was used to compare the condition indices of infected and uninfected mussels. To analyse the possible independent effects of temperature, oxygenation and salinity on the occurrence of infection, we used the binary logistic regression. The chi-square test of independence was employed to examine the occurrence of simultaneous infections with *E. inquilina* and intracellular ciliates.

## Results

### 1. Occurrence of *Eugymnanthea inquilina* in Slovenian Mediterranean mussels

No increased mortality was detected in shellfish farms during the one year sampling period.

The length of the mussels varied from 5.0 to 9.8 cm (average 7.0 cm).

The total weight of mussels varied from 5.0 to 39.5 g (average 15 g) whereas the weight of the flesh varied from 1.2 to 12.2 g (average 4.15 g). The condition index varied from 11.17 to 69.33 (average 28.14).

Overall, the prevalence of hydroid infection was 60.6%. The hydroids (Figure 1) were 111–664  $\mu\text{m}$  long (average 345  $\mu\text{m}$ ). They were either attached to the mantle epithelium or found inside the mantle cavity (Figure 1). No alterations were observed at the point of attachment.

The average condition index of infected mussel was 28.3 and it was lower than that of healthy ones (29.8). The difference in the condition index between infected and healthy mussels was statistically significant ( $t = 4.095$ ;  $p < 0.001$ ).

## 2. Correlation with environmental parameters

The measured sea temperatures, oxygenation and salinity in relation to hydroid prevalence are presented in figure 2.

The highest average prevalence of infection (82.7%) was detected in summer (June, July, August) with an average sea temperature of 24.1  $^{\circ}\text{C}$ , oxygenation of 7.6 mg/l and salinity of 38.1‰. The lowest prevalence (37.0%) was observed in winter (December, January, February) with an average

sea temperature of 9.1  $^{\circ}\text{C}$ , average oxygenation of 11.6 mg/l and average salinity of 37.25‰. The highest absolute prevalence was detected in September (86.7%) with a sea temperature of 20.3  $^{\circ}\text{C}$ , oxygenation of 6.9 mg/l and salinity of 39‰, and the lowest in March (20%) with the sea temperature of 11.1  $^{\circ}\text{C}$  and salinity of 32.5‰.

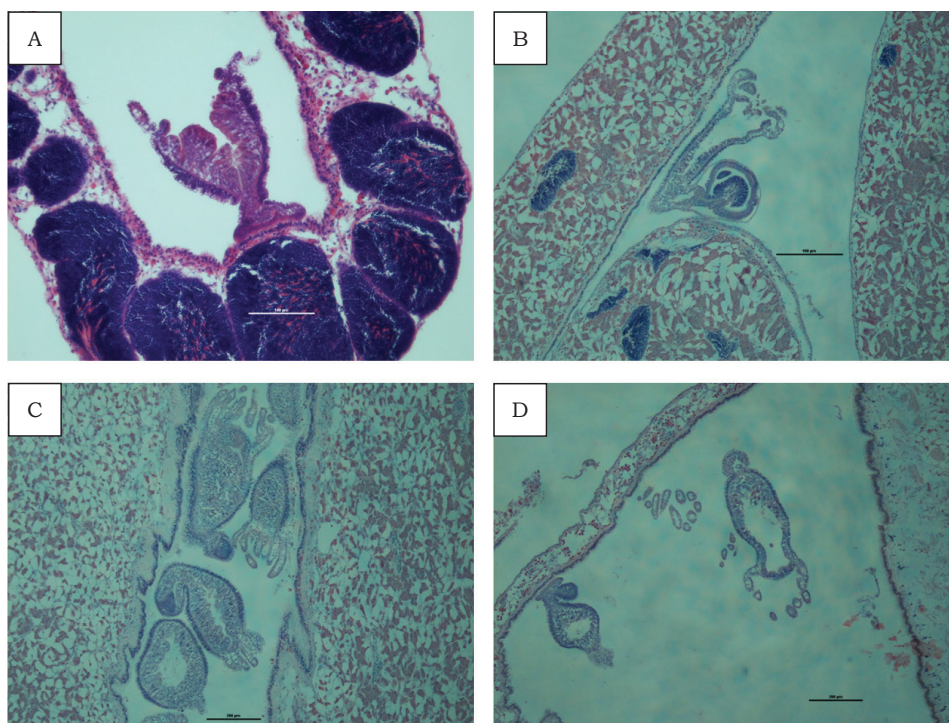
The differences in the infection prevalence between the months of sampling were statistically significant ( $V = 0.445$ ;  $p < 0.001$ ). The binary logistic regression showed that oxygen content and salinity have a statistically significant ( $p < 0.001$ ) correlation with the presence of infection, while temperature does not (Table 1).

## 3. Concurrent infection with intracellular ciliates of mussels

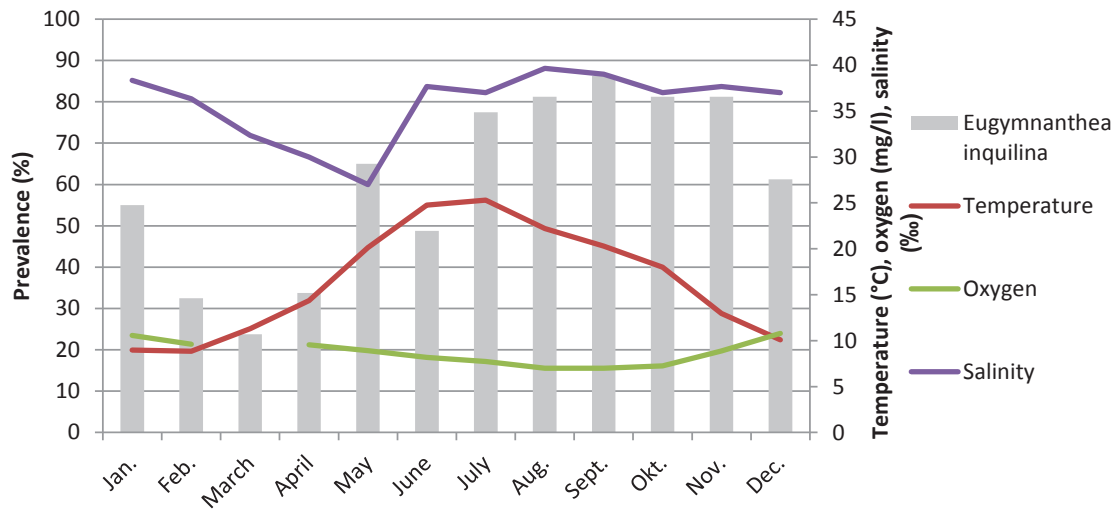
The occurrence of intracellular ciliates of mussels and *E. inquilina* in all the sampled mussels is summarised in Table 2.

A chi-square test of independence was performed to examine the relation between the occurrence of intracellular ciliates in mussels with or without hydroids. The relation between the variables was significant,  $\chi^2 (1, N = 960) = 5.96$ ,  $p = 0.015$ . Intracellular ciliates are less likely to invade mussels with *Eugymnanthea inquilina*.

**Figure 1:** *Eugymnanthea inquilina* in the mantle cavity of Mediterranean mussels. A: *E. inquilina* polyp attached to the mantle, HE staining,  $\times 100$ ; B: *E. inquilina* polyp with a medusoid bud surrounded by a membrane in the mantle cavity, HE staining,  $\times 40$ ; C: Numerous *E. inquilina* polyps in the mantle cavity, HE staining,  $\times 40$ ; D: Two *E. inquilina* polyps, one with a medusoid bud attached to the mantle, another in the mantle cavity, HE staining,  $\times 40$







**Figure 2:** Prevalence of hydroids in different months in correlation with sea temperature, salinity and oxygenation

**Table 1:** Results of the binary logistic regression analysing the possible independent effects of temperature, oxygenation and salinity on the occurrence of infection.

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1(a)	T <sup>a</sup>	,001	,018	,003	1	,960	1,001
	O <sup>b</sup>	-,335	,082	16,512	1	,000	,715
	S <sup>c</sup>	,060	,017	12,481	1	,000	1,062
	Constant	1,329	1,331	,997	1	,318	3,777

<sup>a</sup> Temperature <sup>b</sup>Oxygen concentration; <sup>c</sup>Salinity

**Table 2:** The observed numbers of Mediterranean mussels infected with *Eugymnanthea inquilina* and intracellular ciliates of mussels

Intracellular ciliates	<i>Eugymnanthea inquilina</i>		Total
	Yes	No	
Yes	126	108	234
No	456	270	726
Total	582	378	960

**Discussion**

Our research is the first to demonstrate that *Eugymnanthea inquilina* is present in cultured Mediterranean mussels from the Slovenian sea. The average incidence of infection with the hydroid was 60.6%. This is comparable to the prevalence of 73.9% reported in the Ionian Sea, Italy (3) and 48.3% (2) and up to 78.1% (7) in the North Aegean Sea, Greece.

We observed statistically significant differences in the prevalence of infection with *Eugymnanthea*

*inquilina* in different seasons. The highest prevalence was observed during summer and autumn months, which is contradictory to the data reported in Rayyan et al. (2) and Piraino et al. (3), who, for the same part of the year, both found a decrease in prevalence. Oxygen content and salinity were shown to have a statistically significant ( $p < 0.001$ ) correlation with the presence of infection with *E. inquilina*, while temperature did not. Increased salinity and decreased oxygen are probably stressful conditions for the host, which could explain the increased prevalence of the medusoid.

Since no increased mortality occurred in shellfish farms during the year of sampling, we can conclude that the effects of *Eugymnanthea inquilina* on the host organism are not lethal. The condition index of invaded mussels was significantly lower than that of healthy ones. This implies that serious invasions of *Eugymnanthea inquilina* in mussel farms could mean a decrease in the production and revenues of the farms.

The mussels infected with *Eugymnanthea inquilina* were less likely to contain intracellular ciliates than hydroid-free mussels. Our previous research (9) has shown that the difference in the condition indices between mussels infected with ciliates and healthy ones was not statistically significant. However, ciliates were most often observed in longer and heavier mussels. We concluded that intracellular ciliates more frequently inhabit older mussels in better condition and are harmless commensals when present in small numbers, but a more numerous infection causes a decrease in the mussel's condition index. The observed decrease in the occurrence of intracellular ciliates in mussels infected with *Eugymnanthea inquilina* could therefore be an indirect consequence of decreased condition index of the mussels. It is also possible that *Eugymnanthea inquilina* has some direct effect on the presence of intracellular ciliates of mussels (e.g. by excreting repellent substances), but this possibility needs to be researched further.

On the basis of our research we can conclude that *Eugymnanthea inquilina* is present in Slovenian shellfish farms to a relatively large extent. The interaction between the host and endobiont is not mere commensalism since infection with *E. inquilina* decreases the condition of affected mussels. Similar to some of the other authors (2, 7), we can conclude that the relationship between Mediterranean mussels and hydroids is more likely to be parasitism. An increase in the prevalence of *Eugymnanthea inquilina* in shellfish farms could therefore potentially represent a threat to their production. It is recommended that the condition of the infection be monitored in the future in order to have a comprehensive warning system for potential decline in the condition and growth of *Mytilus galloprovincialis* in the Slovenian sea.

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## POJAVLJANJE IN UČINKI TRDOŽIVNJAKA *EUGYMNANTHEA INQUILINA* V GOJENIH MEDITERANSKIH KLAHAVICAH (*MYTILUS GALLOPROVINCIALIS*) V SLOVENIJI

S. Žižek, M. Gombač, M. Pogačnik

**Povzetek:** Mediteranska klapavica (*Mytilus galloprovincialis*) je v slovenskem morju najštevilčnejša in najpogosteje gojena vrsta školjk. Gojijo jo na treh lokacijah, pri Seči, Strunjanu in Debelem Rtiču. V našo raziskavo smo vključili 960 klapavic, ki smo jih naključno vzorčili v obdobju med novembrom 2007 in oktobrom 2008 v školjčičih pri Seči in Strunjanu. Med vsakim vzorčenjem smo izmerili temperaturo morja, koncentracijo raztopljenega kisika in slanost vode. Školjke smo izmerili in stehtali, jim izračunali kondicijski indeks in jih s histopatološko preiskavo pregledali na prisotnost trdoživnjakov. Med vzorčenjem v školjčičih nismo opazili poginov školjk. Trdoživnjaka *Eugymnanthea inquilina* smo diagnosticirali v 60,6 % školjk, največjo prevalenco smo zaznali poleti. Trdoživnjaki so bili pritrjeni na plašč ali pa smo jih našli proste v plaščevi votlini. Na mestu pritrditve nismo opazili nobenih tkivnih sprememb. Razlike v prevalencah v različnih letnih časih so bile statistično značilne. Koncentracija raztopljenega kisika in slanost sta značilno vplivali na prisotnost trdoživnjakov, temperatura pa ne. Povprečen kondicijski indeks klapavic s trdoživnjaki je bil statistično značilno nižji od kondicijskega indeksa zdravih klapavic, kar kaže na možnost parazitizma trdoživnjaka *Eugymnanthea inquilina*.

To je prvo poročilo o prisotnosti endobiontskega trdoživnjaka *E. inquilina* v mediteranskih klapavicah v slovenskem morju.

**Ključne besede:** *Eugymnanthea inquilina*; *Mytilus galloprovincialis*; histopatologija; kondicijski indeks; Tržaški zaliv



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- ustvarjanje novih delovnih mest,
- povečanje produktivnosti in dobičkonosnosti,
- visoko motiviran in usposobljen kader s primernim vodenjem, kar zagotavlja
- kakovost izvajanja storitev,
- postati vodilno podjetje, ki ponuja rešitve, ki podjetju omogočajo da si na enem
- mestu zagotovi vse dejavnosti, ki ne spadajo v njegovo osnovno dejavnost.

## Prednosti poslovanja z nami:

- vse svoje potrebe in vizije uresničite s klicem na eno telefonsko številko,
- razbremenite se ukvarjanja z obrobnimi zadevami,
- posvetite se svojemu strokovnemu delu,
- informacijska tehnologija,
- prilagodljivost,
- zanesljivost,
- povečanje dobičkonosnosti,
- zmanjšanje stroškov dela,
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- visoka profesionalizacija,
- visoka strokovnost,
- visoka uspešnost,
- konkurenčne cene,
- vse na enem mestu.



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